BIOCONVERSION OF MOUNTAIN PINE BEETLE-KILLED LODGEPOLE PINE TO ETHANOL

by

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ABSTRACT

Global warming is widely acknowledged as being caused by an increase in the production of greenhouse gases, largely CO_2, generated from the burning of fossil fuels such as gasoline. By offsetting a portion of gasoline usage with alternative fuels, such as ethanol, CO_2 emissions could be reduced. Bioethanol can be produced from many types of biomass, including softwoods. For maximal ethanol yield from softwoods, the most effective bioconversion process is considered by many to be SO_2-catalysed steam explosion followed by separate or simultaneous hydrolysis and fermentation. This method has been shown to be effective on both spruce and Radiata pine, although past research at UBC on Douglas-fir showed that additional delignification was necessary in order to obtain satisfactory sugar recovery and hydrolytic conversion. Another promising softwood species for bioconversion in British Columbia is lodgepole pine, which has been severely affected by the mountain pine beetle and its associated fungus and is consequently widely available and relatively inexpensive.

Prior to investigating the bioconversion of lodgepole pine, the UBC steam explosion process was evaluated and was found to be comparable to that used at Lund University in Sweden. Following this, optimization of pretreatment conditions of beetle-killed lodgepole pine for maximal ethanol yield revealed that the highest ethanol yield after SSF, 77% of theoretical, was derived from substrates pretreated at 200°C, 5 min, 4% SO_2. When these and other conditions were applied to healthy, and mixed (50/50 healthy/beetle-killed) wood, the beetle-killed substrate provided higher sugar recovery after pretreatment, higher hydrolytic conversion, and higher overall ethanol yield after SSF. This was likely a result of two factors. The first, reduced uptake of SO_2 during the

impregnation step effectively reduced the pretreatment severity and increased sugar recovery and fermentability. Secondly, increased fines may have improved the enzymatic digestibility of the substrate.

The work in this thesis established the technical feasibility of producing ethanol from lodgepole pine in a two-step process consisting of SO_2-catalyzed steam explosion followed by SSF of the combined liquid and solid fractions. In addition, the short residence time required for SSF as well as the reduced cost of the raw material suggest that the process could be economically attractive as well.

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%	percent
µl	microlitre
BK	beetle-killed
CAD	Canadian dollar
CBU	cellobiase unit
cm	centimetres
CO_2	carbon dioxide
CS	combined severity
DP	degree of polymerization
FPU	filter paper unit
g	grams
GC	gas chromatography
H	high severity
h	hour
H_2SO_4	sulphuric acid
HHF	hybrid hydrolysis and fermentation
HMF	5-hydroxymethyl furfural
HPLC	high performance liquid chromatography
l	litre
L	low severity
R_o	severity factor
LPP	lodgepole pine
M	medium severity
M	molar
m.c.	moisture content
$MgSO_4 \cdot 7H_2O$	magnesium sulphate heptahydrate
min	minute
mL	millilitre
mm	millimetre
mM	millimolar
NaOH	sodium hydroxide

$(NH_4)_2HPO_4$	ammonium phosphate
nm	nanometer
°C	degree Celsius
OD	optical density
ODT	oven-dried ton
rpm	revolutions per minute
s	second
SHF	separate hydrolysis and fermentation
SO_2	sulphur dioxide
SSF	simultaneous saccharification and fermentation
SSL	spent sulphite liquor
T	temperature
t	time
TAPPI	Technical Association of the Pulp and Paper Industry
UBC	University of British Columbia
USD	United States dollar
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight
WIF	water-insoluble fraction
WSF	water-soluble fraction
β	beta

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1 INTRODUCTION

1.1 Overview

Anthropogenic climate change brought on by increased CO_2 emissions has encouraged countries, companies, and researchers around the world to look for environmentally friendly fuels which will slow the rate at which the planet is heating up. Ethanol produced from biomass has been at the forefront of this research for a number of reasons: it is clean-burning, renewable, and can be blended with gasoline and used in conventional automobile engines (Lynd 1996; Wheals, et al. 1999; Zaldivar, et al. 2001). While many types of biomass can be utilized, lignocellulosic feedstocks such as crop residues, hardwoods and softwoods are attractive since they are often available as waste from other processes, such as agriculture and saw milling operations (Lin and Tanaka 2006; Lynd 1996). As a result, these materials are sometimes inexpensive and in low demand for other applications. While the basic components of all lignocellulosic materials are cellulose, hemicellulose, and lignin, softwoods and hardwoods usually contain more lignin than agricultural residues. In general, the hemicellulose component of softwoods is less acetylated than hardwoods, and in combination with a less reactive type of lignin, softwoods consequently tend to be the most challenging feedstock to convert to ethanol (Galbe and Zacchi 2002). Despite the challenges they present, softwoods are highly abundant in Northern countries like Canada and Sweden and many processes are being developed to efficiently convert this wood to ethanol. Typically the three main steps that are critical for achieving a high ethanol yield in most processes are pretreatment, hydrolysis and fermentation (Duff and Murray 1996; Galbe and Zacchi 2002). SO_2-catalysed steam explosion is one of the few pretreatment processes that is severe enough

to solubilize hemicellulose, strip away lignin, and render the cellulose digestible by cellulytic enzymes while minimizing the production of degradation derived compounds that inhibit fermentation.

Early work on SO_2-catalysed steam pretreatment of softwoods utilized Radiata pine and spruce (Clark and Mackie 1987; Schwald, et al. 1989). Both species could be treated with moderately severe conditions and resulted in over 80% hydrolytic conversion of the cellulose and high overall sugar recovery. More recently, pretreatment of mixed spruce and pine at moderate conditions generated a substrate that provided 79% conversion after hydrolysis and 88% of the theoretical ethanol yield (Stenberg, et al. 1998). In contrast to the relative ease of pretreatment of spruce and pine, past work on bioconversion of Douglas-fir at UBC has been less successful. After pretreatment at moderately severe conditions, only 24% hydrolytic conversion and 77% sugar recovery could be achieved (Wu, et al. 1999). With delignification of the pretreated insoluble fraction, hydrolytic conversion could be increased dramatically to 100% (Pan, et al. 2004; Yang, et al. 2002). However, fermentation of the delignified hydrolysate and water-soluble fraction generally resulted in low ethanol concentrations (Robinson, et al. 2003), further reducing the economic feasibility of a delignification step. It has been suggested that the recalcitrance of Douglas-fir observed during bioconversion is due to its dense and rigid structure (Green, et al. 1999).

Lodgepole pine is another major softwood species found in British Columbia (COFI 1999). Recently the lodgepole pine forests of BC have been subject to a widespread outbreak of the mountain pine beetle, which has killed over 283 million cubic metres of timber (BC Ministry of Forests 2005). Together with sapstaining fungi, the beetle

generally kills the tree within a year of attack. The resulting timber is only suitable for pulp and paper and structural lumber applications for a short period of time before fibre loss, decay, and checking due to drying render the wood unusable. Compared to healthy wood, beetle-killed wood is generally thought to be chemically similar, with some structural differences brought on by the drying out of the tree such as reduced permeability and increased production of fines during processing (Nebeker, et al. 1993; Thomas 1985). Bioconversion could be an ideal application for this wood.

The work in this thesis aimed to evaluate the suitability of lodgepole pine for ethanol production. The first step was to optimize the pretreatment conditions for maximal ethanol yields from beetle-killed lodgepole pine by selecting conditions that provide high sugar recovery and high hydrolytic conversion. However, in order for the process to be economically viable, delignification should not be required. In addition, hydrolysis and fermentation should be completed with as short a residence time and as little processing as possible. After selection of the optimum conditions, our goal was to examine whether beetle-killed wood behaves any differently from healthy lodgepole pine in terms of sugar recovery, hydrolytic performance and overall ethanol yield. The bioconversion behaviour of both beetle-killed and healthy pine could then be compared to that of spruce at Lund University and Douglas-fir at UBC in order to determine how lodgepole pine measures up to other softwoods.

1.2 Relevance of bioethanol and bioconversion

1.2.1 Fossil fuels and global warming

Global warming is widely recognized as being at least partially accelerated by increasing emissions of CO_2, largely from the burning of fossil fuels (Crowley 2000). Oil reserves

around the world are being depleted, and as result of this, as well as political instability, prices at the time of writing have risen above $70/barrel. The price of oil is expected to continue to rise as it becomes increasingly difficult to extract enough oil to meet the demands of a rapidly industrializing world (Kerr 2005). It has been suggested that fuels produced from biomass (biofuels) can help to reduce reliance on oil. 1.3 billion tonnes of biomass are available annually in the U.S. from forestland and agricultural land, enough to produce sufficient biofuels to displace a third of the fossil fuel consumption in the US (Perlack, et al. 2005). Currently, ethanol makes up 99% of biofuel production (Farrell, et al. 2006), since it can readily be used in place of gasoline. Ethanol can be blended with gasoline and used in conventional engines at up to 10% ethanol, or in modified flex-fuel engines at blends of up to 85% ethanol (Lynd, et al. 1991; Wheals, et al. 1999).

1.2.2 Bioethanol

As a fuel, ethanol provides considerable benefits over gasoline. Combustion of ethanol in fuel results in up to a 12% reduction in energy-specific CO_2 emissions (g CO_2 per BTU) (Chang, et al. 1991). Since this doesn't include the benefit derived from using plant matter, which absorbs as much CO_2 while living as it emits when combusted, combustion of ethanol can provide a net reduction in CO_2. In addition, emission of the toxic compounds formaldehyde, benzene and 1,3-butadiene is reduced, although acetaldehyde emissions are increased (Chang, et al. 1991). As modern catalytic converters can remove these compounds (Duff and Murray 1996), there is a net reduction in emissions of harmful compounds when ethanol is used as a fuel. In terms of energy, ethanol has only two thirds of the volumetric energy content of gasoline. However, it is about 15% more efficient than gasoline in optimized engines, meaning that it is possible to travel 75-80%

as far on a given volume of pure ethanol compared to the same volume of gasoline (Bailey 1996; Wyman 1996). Since domestic feedstocks can be used to produce bioethanol, the price fluctuations and political instability associated with petroleum are removed. For instance, the country of Brazil independently produces enough bioethanol, 16 billion litres per year, to provide 40% of its driving fuel (Orellana and Neto 2006).

1.3 The bioconversion process

Bioconversion of lignocellulosic material to ethanol typically requires that the feedstock be pretreated prior to hydrolysis and fermentation (Boussaid, et al. 2000; Chang, et al. 1998; Holtzapple, et al. 1991; Stenberg, et al. 1998). Pretreatment serves to fractionate the substrate into cellulose, hemicellulose and lignin and improve the digestibility of the cellulose for subsequent enzymatic hydrolysis. In the final step, hemicellulosic sugars and the enzymatic hydrolysate are fermented to ethanol (Figure 1-1). For an efficient and productive fermentation, the hydrolysate should contain a high concentration of sugars and minimal amounts of inhibitors. Pretreatment is thus an important determinant of the downstream success of both hydrolysis and fermentation.

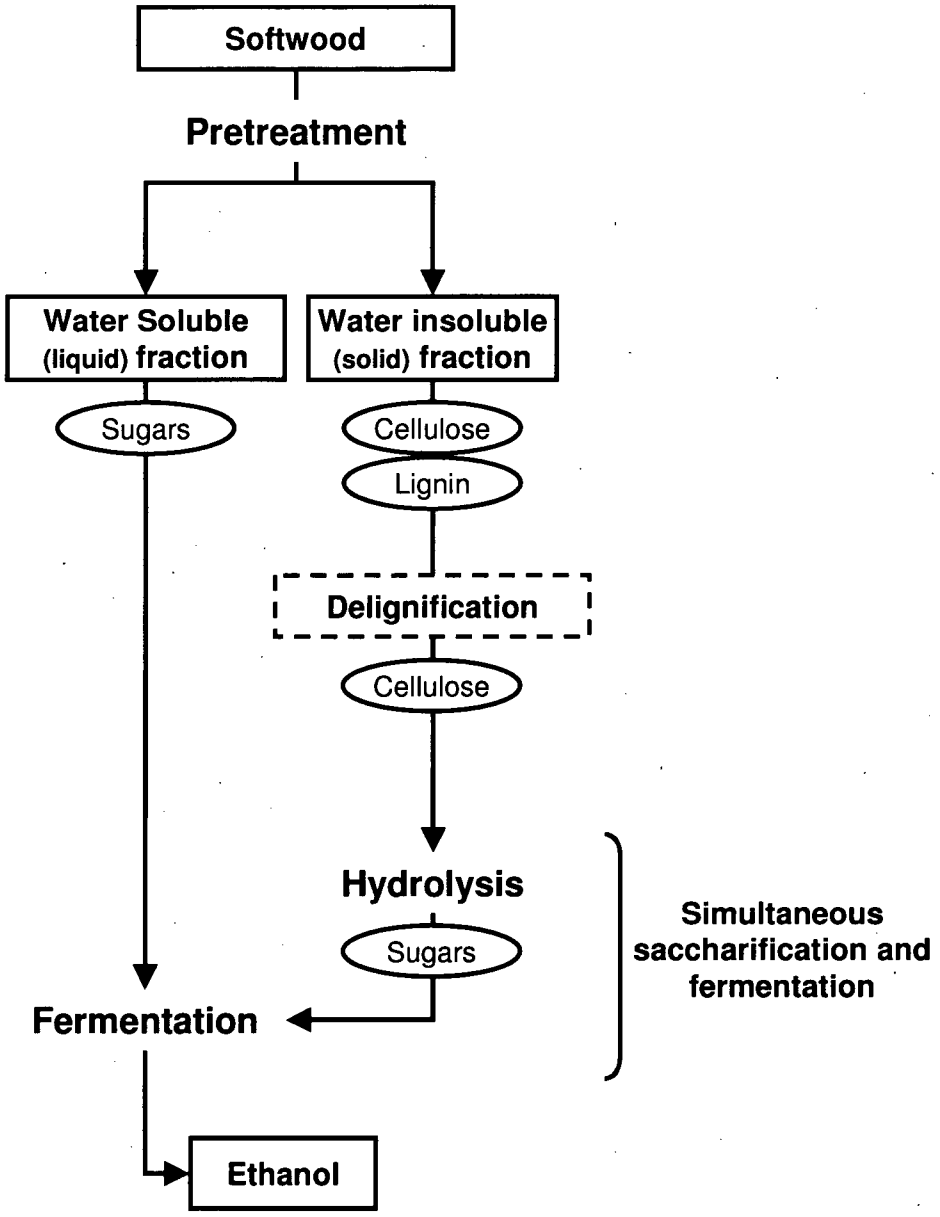

Figure 1-1. Bioconversion of softwoods to ethanol using acid catalyzed steam explosion. The delignification step shown is optional, and only required on certain substrates.

1.3.1 Feedstock structure and composition

By definition, lignocellulosic materials are those that contain predominantly lignin, cellulose, and hemicellulose. Each of these three components is made up of smaller subunits, carbohydrates in the case of cellulose and hemicelluose, and aromatic groups for lignin. The makeup of these subunits, as well as the ratio of lignin: cellulose: hemicellulose define each type of feedstock (Table 1-1). Plant cell walls typically share the same structure, with cellulose forming the "skeleton" of the cell, surrounded by a matrix of hemicellulose and encrusted with lignin (Sjöström 1993). In general, agricultural residues contain less lignin than wood, with hardwood containing less than softwood. While cellulose is solely made up of glucan, the structure can vary considerably from amorphous to highly crystalline (Sjöström 1993). Hemicellulose is made up of a number of carbohydrates, including xylose, arabinose, galactose, mannose, and glucose. The proportions and linkages of each vary considerably between different types of biomass (Sjöström 1993).

Table 1-1. Composition of various lignocellulosic feedstocks (Ghosh and Singh 1993; Sun and Cheng 2002).

	Composition (%)		
	Lignin	Cellulose	Hemicellulose
Softwood	25-35	45-50	25-35
Hardwood	18-25	40-55	24-40
Agricultural residues	10-30	25-45	10-40

Cellulose is a homopolysaccharide composed of chains of glucose units linked together by β-(1-4) glycosidic bonds (Sjöström 1993). The number of glucose units in each chain or molecule of cellulose is known as the degree of polymerization (DP), although it has been shown that the basic structural unit is actually cellobiose (Blackwell 1982).

Cellulose chains can aggregate and connect via inter-molecular hydrogen bonds to form units known as elementary fibrils that contain ordered, crystalline, regions as well as disordered, amorphous regions (Sjöström 1993). Crystalline regions are generally more resistant to enzymatic attack, while amorphous regions present more accessible sites (Eriksson, et al. 1990).

Hemicellulose shows much more chemical variability between different types of biomass than cellulose. Hardwoods and agricultural residues contain highly acetylated glucuronoxylan and small amounts of glucomannan, while softwoods are made up of galactoglucomannans and partly acetylated arabinoglucuronoxylans (Sjöström 1993). The pentan-rich hemicellulose found in agricultural residues and hardwoods is thus more susceptible to acid autohydrolysis than hexan-rich softwood hemicellulose (Grohmann, et al. 1989; Kong, et al. 1992).

Compared to softwoods, hardwoods have less lignin overall, with their lignin consisting of predominantly guaiacyl-syringyl units (Campbell and Sederoff 1996). Softwood lignin is composed of primarily guaiacyl, which is known to restrict fibre swelling and enzyme permeability more than syringyl lignin due to increased methoxylation (Ramos, et al. 1992). Guaiacyl lignin is also known to condense during steam pretreatment, reducing the accessibility for subsequent enzymatic hydrolysis (Shevchenko, et al. 1999). These differences in lignin composition as well as differences in distribution in and between cells likely account for the increased recalcitrance of softwoods compared to hardwoods.

In addition to carbohydrates and lignin, lignocellulosics contain a variety of extractives including phenols such as tannins, terpene alcohols, ketones, and resin components such as fatty acids, alcohols, resin acids, and phytosterols (Fan, et al. 1982; Sjöström 1993).

1.3.2 Pretreatment methods

The wide variety of pretreatment methods available can be classified as physical, chemical and biological or a combination thereof (Hsu 1996). Pretreatment is necessary to improve the accessibility of the cellulose for hydrolysis by removing or modifying the surrounding hemicellulose and lignin. Physical methods include mechanical processes such as ball milling, attrition, and wet disk refining (Hsu 1996), hydrothermolysis, pyrolysis (Shafizadeh and Stevenson 1982), irradiation using electron beams and other high-energy radiation (Fan, et al. 1982) as well as microwave heating (Ooshima, et al. 1984). Uncatalyzed steam explosion is possible for some feedstocks, and is known as autohydrolysis (Brownell and Saddler 1987; Ramos 2003). The subsequent breakdown of glycosidic linkages is dependent on acids formed within the biomass itself (Ramos 2003). Chemical pretreatments include ammonia (Holtzapple, et al. 1991), solvent (Pan, et al. 2005), wet oxidation (McGinnis, et al. 1983), alkali (Chang, et al. 1998), acid (Torget, et al. 1990) and others. Chemicals can also be used to catalyze physical pretreatments, as in the case of pH-controlled hydrothermolysis (McMillan 1994) and acid-catalyzed steam explosion (Tengborg, et al. 1998).

Biological treatments make use of the agents of wood decay in nature; bacteria, fungi, and soil microflora (Ghosh and Singh 1993). However, for effective pretreatment for bioconversion purposes, it is important that minimal sugars be consumed and only the lignin affected. The primary organisms that degrade wood are white rot, brown rot, and soft rot fungi. Of theses, the only ones that degrade primarily lignin are the white rot fungi, of the kingdom Basidiomycetes (Ghosh and Singh 1993). Ferraz et al. found that treatment of Radiata pine chips with white rot fungi was effective in removing as much

as 30% of the lignin in 90 days (2001). While there is no energy input required during this time period, it is considered too long to be technically and economically feasible (Hsu 1996).

1.3.2.1 Softwood pretreatment: SO_2-catalyzed steam explosion

The composition and structure of softwoods requires pretreatment strategies different from those used on hardwoods or other lignocellulosics. The structure of softwood is more rigid and lignin content is higher. Due to these unique characteristics, only a few pretreatment processes have led to effective bioconversion of softwood. These include organosolv (Kurabi, et al. 2005; Pan, et al. 2005), dilute-acid (Nguyen, et al. 2000; Söderstrom, et al. 2003), and SO_2-catalyzed (Boussaid, et al. 2000; Clark and Mackie 1987; Stenberg, et al. 1998) steam explosion. Organosolv effectively separates cellulose from lignin, but most of the hemicellulosic sugars are lost during the multi-stage process. However, the lignin isolated during the process is in a form that can be potentially utilized for a variety of industrial products, such as polymer fibres and adhesives (Cetin and Ozmen 2002; Kubo and Kadla 2005; Pan, et al. 2005).

An acid catalyst is required for effective steam explosion of many softwood feedstocks due to the fact that the amount of acetylated groups is much lower than in hardwoods, meaning that the extent of non-catalyzed autohydrolysis during steam explosion is much less (Galbe and Zacchi 2002). The most commonly used acid catalysts are liquid H_2SO_4 and gaseous SO_2. Both require the feedstock to be impregnated for a period of time with the acid prior to pretreatment, and their effectiveness depends on the feedstock and conditions used. However, SO_2 is easier and faster to introduce, and also results in reduced steam consumption (Schwald, et al. 1989). Studies comparing SO_2 to H_2SO_4

have found that impregnation with SO_2 provides approximately the same sugar yields after pretreatment, but the resulting substrate is more readily fermented due to the presence of fewer fermentation inhibitors (Larsson, et al. 1999; Tengborg, et al. 1998). For these reasons, SO_2-catalysed steam explosion is commonly used for softwood pretreatment.

1.3.2.1.1 Pretreatment severity

The extent of pretreatment during SO_2-catalyzed steam explosion is dictated by three factors: residence time in the reactor, temperature, and concentration of SO_2. The levels of each of these three factors determine how severe the pretreatment is. Severity can be quantified by calculating the severity factor R_o (Eq. 1), where t is time in seconds and T is temperature in degrees Celsius (Overend, et al. 1987).

$$R_o = te^{(T-100)/14.75} \qquad \textbf{(Eq. 1)}$$

Since this factor takes into account only time and temperature and not acid concentration, there is not always a direct correlation between the degree of pretreatment and R_o. The combined severity (CS) (Eq. 2) factors in the acid concentration, and is more suitable for comparison of acidic pretreatments to other pretreatments (Chum, et al. 1990). However, for steam explosion catalyzed by SO_2, the pH of the reaction mixture before pretreatment is difficult to measure accurately, so the combined severity factor is not often used.

$$CS = \log R_o - pH \qquad \textbf{(Eq. 2)}$$

As pretreatment severity on a given substrate increases, hemicellulosic sugars are the first to be affected due to their low degree of polymerization and amorphous structure (Sjöström 1993). As severity increases further, cellulose begins to break down. By separating pretreatment into two steps of increasing severity, the sugar recovery can be maximized. The first step, at relatively mild severity, allows recovery of most

hemicellulosic sugars. Higher severity conditions used for the second step allow for degradation and partial hydrolysis of cellulose, reducing the amount of enzyme required during subsequent enzymatic hydrolysis (Nguyen, et al. 2000; Söderstrom, et al. 2003). The drawback to this method is that there is dilution of the sugars (Taherzadeh, et al. 1997), reducing the potential concentration of ethanol after fermentation, as well as the increased production of fermentation inhibitors during the second, higher severity step (Larsson, et al. 1999).

1.3.3 Hydrolysis and fermentation

1.3.3.1 Enzymatic hydrolysis

In nature, many different microorganisms produce extracellular enzymes that hydrolyse cellulose. However, few of these organisms are able to digest the highly crystalline cellulose present in pretreated softwoods. Those that have been investigated include the fungi *Trichoderma*, *Penicillium*, and *Aspergillus* (Eriksson, et al. 1990). While these organisms produce a number of different glycolytic enzymes, there are three primary activities necessary to hydrolyse cellulose. Endo-1,4-β-glucanases randomly cleave β-1-4 glycosidic linkages over the length of the chain, while exo-1,4-β-glucanases cleave off either glucose or cellobiose from reducing and non-reducing ends. Cellobiose is produced in higher amounts than glucose, and is subsequently hydrolysed to glucose by 1,4-β-glucosidases (Eriksson, et al. 1990). Since cellobiose is highly inhibitory to cellulase enzymes, supplemental 1,4-β-glucosidase is often added to compensate for low levels of this enzyme in the cellulase complex (Holtzapple, et al. 1990). Other inhibitors of cellulase enzymes include ethanol (Wu and Lee 1997) and glucose (Holtzapple, et al.

1990). End product inhibition by glucose and cellobiose prevents hydrolysis of cellulose at high soluble sugar concentrations.

Structurally, the main factors that limit hydrolysis are the structure of the cellulose and the effect of lignin (Fan, et al. 1982). As crystallinity increases, the cellulose contains fewer reducing ends, limiting the number of sites that can be acted on by endoglucanases (Sun and Cheng 2002). Lignin surrounds the cellulose and blocks potential binding sites as well as irreversibly binding cellulase enzymes (McMillan 1994; Sun and Cheng 2002).

1.3.3.2 Fermentation

A number of microorganisms are capable of fermentation of sugars to ethanol. Anaerobic thermophilic bacteria and filamentous fungi have been shown to convert cellulose to ethanol (Lin and Tanaka 2006). However, this process is generally slow (3-12 days) and provides low ethanol yields, likely due to the inability of these organisms to survive in increasingly ethanol-rich environments. In addition, undesirable by-products such as acetic acid and lactic acid are often generated (Lin and Tanaka 2006). In order to circumvent these shortcomings, yeast are often utilized for fermentation. Pentose fermenting organisms such as *Pichia stipitis*, *Pachysolen tannophilus* and *Candida shehatae* are well suited for hardwoods and agricultural residues, since these feedstocks typically contain high concentrations of xylose (Olsson and Hahn-Hägerdal 1996). Softwoods have relatively low levels of pentose sugars, only 6-7% of the total wood (Galbe and Zacchi 2002), and are often fermented with *Saccharomyces cerevisiae*, which ferments exclusively hexoses.

The ethanologenic properties of *S. cerevisiae* have been known for thousands of years, and it continues to be one of the preferred yeast species due in large part to its hardiness

at high ethanol concentrations (Olsson and Hahn-Hägerdal 1993). While *S. cerevisiae* possesses many desirable traits, improving the conditions that it can tolerate can maximize ethanol productivity and yield. The yeast's main limitations are its low tolerance to some inhibitory compounds generated during pretreatment, and its inability to ferment most sugars with the exception of glucose. To reduce sensitivity to inhibitors, yeast can be acclimated to the inhibitors by growing them on the water-soluble fraction prior to fermentation (Alkasrawi, et al. 2006). Certain strains of *S. cerevisiae* can be selected which ferment alternative sugars such as galactose (Keating, et al. 2004b) or grow particularly well on pretreatment hydrolysates (Alkasrawi, et al. 2006). Alternatively, strains can be selected which thrive at higher temperatures, allowing simultaneous saccharification and fermentation without a compromise in temperature (Szczodrak and Targonski 1988). Genetic modification is another means of improving the yield or rate of ethanol production by engineering strains of *S. cervisiae* which effectively ferment pentoses (Jeffries and Shi 1999), produce glycolytic enzymes (Penttila, et al. 1988; Van Rensburg, et al. 1998), or metabolize inhibitors (Liu, et al. 2005).

1.3.3.3 Separate hydrolysis and fermentation & simultaneous saccharification and fermentation

Hydrolysis and fermentation can be carried out separately or simultaneously, and there are advantages and disadvantages for each method. The primary differences between the processes of fermentation and hydrolysis are the optimum temperature and, to a lesser extent, pH, for the enzyme and fermenting organism. Separate hydrolysis and fermentation (SHF) allows each process to run at the optimum temperature and avoids inhibition of enzymes by ethanol, although end product inhibition can reduce the rate and

extent of hydrolysis. Simultaneous saccharification and fermentation (SSF) solves the problem of end product inhibition since sugars are fermented as soon as they are produced. However, a compromise in temperature is required since *S. cerevisiae* requires that the temperature is kept below 40°C for efficient fermentation (Eklund, et al. 1990). However, the lack of glucose inhibition can more than make up for the reduced hydrolysis temperature, as it has been observed that cellulose hydrolysis rates can be increased by 13-30% when using SSF rather than SHF (Ghosh, et al. 1982), reducing the amount of enzyme required. Other benefits of SSF can include shorter process time, reduced risk of contamination since glucose is removed and immediately converted to ethanol, and reduced capital costs due to the need for only one vessel (Hinman, et al. 1992). In order to further improve the hydrolytic performance a prehydrolysis step at the enzyme optimum temperature can be added for a period of time prior to adding the yeast and reducing the temperature. This process is known as hybrid hydrolysis and fermentation (HHF) or "non-isothermal SSF" (Sassner, et al. 2006; Varga, et al. 2004)

1.3.3.4 Fermentation inhibition

Steam pretreatment of biomass often leads to the formation of compounds which are inhibitory to yeast, leading to a reduction in productivity or end-product formation (Klinke, et al. 2004). These compounds can be divided as follows: compounds released during pretreatment, sugar degradation products, lignin degradation products, and fermentation products (Olsson and Hahn-Hägerdal 1996).

During pretreatment, acetyl groups associated with the hemicellulose can form acetic acid, which can be inhibitory to *S. cerevisiae* in concentrations above 5 g/l (Taherzadeh, et al. 1997). However, pretreatment of softwood rarely results in such a high

concentration. Extractives such as terpenes, alcohols, phenolics and aromatics like tannins can also be released (Olsson and Hahn-Hägerdal 1996). Other potential inhibitors include metals released from equipment during pretreatment such as chromium, copper, iron and nickel (Olsson and Hahn-Hägerdal 1996) as well as compounds generated from introduced SO_2. These include sulphites, which have been shown to have a mild inhibitory effect on the growth of *S. cerevisiae* (Pilkington and Rose 1988).

Sugar degradation products are important inhibitors of *S. cerevisiae*. Furans such as furfural (from pentoses) (Dunlop 1948) and 5-hydroxymethyl furfural (HMF) (from hexoses) are the products of dehydration reactions during pretreatment under severe acidic conditions (Figure 1-2) (Klinke, et al. 2004). Furan inhibition can often be overcome by assimilation by the yeast. Furfural is normally assimilated by *S. cerevisiae* much faster than HMF, which can take as long as 24 hours (Delgenes, et al. 1996). Inhibitory compounds can also be generated by degradation of lignin. These products include furaldehyde, acetate, hydroxymethylfuraldehyde, syringaldehyde, hydroxybenzaldehyde, and vanillin (Delgenes, et al. 1996). Some of these compounds can be metabolized by *S. cerevisiae*, particularly vanillin and furaldehyde, reducing their inhibitory effects (Delgenes, et al. 1996).

The primary fermentation product in a successful fermentation is ethanol, but for *S. cerevisiae*, a maximum of only 0.51g of ethanol are produced for every gram of sugar consumed (Hahn-Hägerdal, et al. 1994). A large portion of the consumed sugar is lost as CO_2, and anywhere from 5-12% of the assimilated carbohydrate is used for cell growth and maintenance, so the maximum ethanol yield is rarely more than 0.47 g ethanol/g sugar consumed (Lynd, et al. 2002). During fermentation, yeast can produce other

potentially inhibitory compounds, such as acetaldehyde, glycerol, formic, lactic, and acetic acids, 1-propanol, 2-methyl-1-butanol, and 2,3-butanediol (Maiorella, et al. 1983).

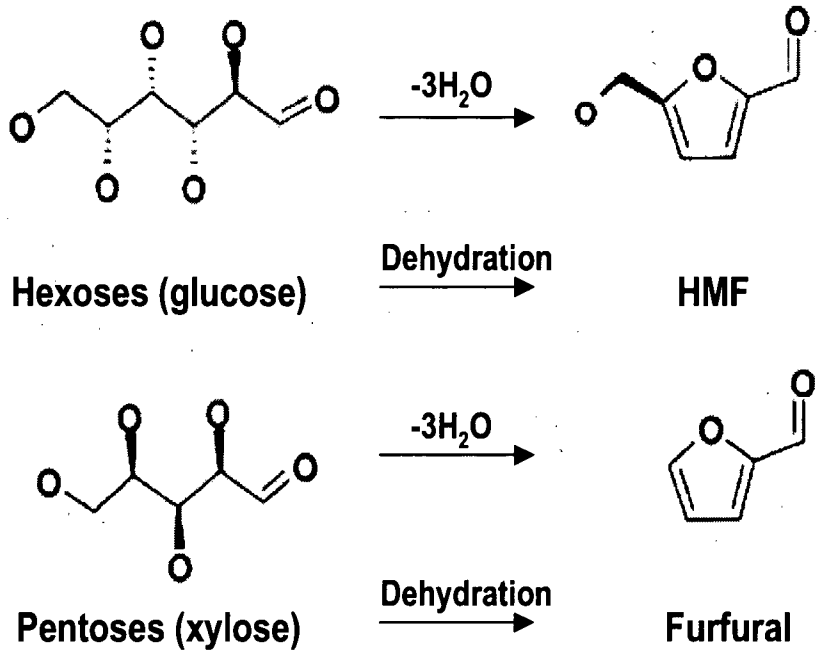

Figure 1-2. Formation of 5-hydroxymethlyl furfural from hexose (above) and furfural from pentose (below). Adapted from Taherzadeh, et al. (2000).

1.3.4 Previous bioconversion work on softwoods

Radiata pine (*Pinus radiata*) was one of the first softwood feedstocks to be investigated using SO_2-catalyzed steam explosion followed by enzymatic hydrolysis. A full optimization revealed that high sugar recovery (88%) and enzymatic hydrolysis (80% conversion) could be achieved with conditions of 215°C, 3 min, and 2.6% SO_2 (Clark and Mackie 1987). However, the resulting substrate was not fermented, so the potential ethanol yields are unknown. Schwald et. al (1989) found that for spruce, the highest sugar recovery and hydrolytic conversion (94% conversion) occurred with pretreatment conditions of 210°C, 2.5 min, and 3.5% SO_2. Other work on mixed spruce and pine showed that conversion of wood pretreated at 205°C, 2 min, and 2% SO_2 to ethanol using *P. stipitis* produced 75% of the theoretical ethanol yield based on sugars in the original material (Wayman, et al. 1986). More recent work on mixed spruce and pine found that pretreatment at 210°C, 2 min, and 3.5% SO_2 generated a substrate which provided 79% conversion after hydrolysis, and 88% of theoretical ethanol yield based on sugars in the raw material (Stenberg, et al. 1998).

Some of the first work at UBC on softwood bioconversion utilized Douglas-fir (*Pseudotsuga menziesii*) as a feedstock (Boussaid, et al. 1997). This wood was chosen due to its high abundance in western Canada and the Pacific Northwest. However, for use in bioconversion, it has been shown to be one of the most refractive softwood species studied. This is likely due to its high density and rigid structure (Green, et al. 1999). Initial optimization of SO_2-catalyzed steam explosion conditions selected for maximum sugar recovery, which resulted in a relatively mild set of conditions (195°C, 4.5 min, 4.5% SO_2) and 77% sugar recovery, but only provided 24% hydrolytic conversion of the

resulting substrate (Wu, et al. 1999). For maximum fermentability, even lower severity conditions (175°C, 7.5 min, and 4.5% SO_2) were selected, providing 85% sugar recovery and 86% of theoretical ethanol production after fermentation of the water-soluble stream (Boussaid, et al. 1999). Conditions which provided the best hydrolytic conversion along with good sugar recovery were 195°C, 4.5 min, 4.5% SO_2, but hydrolytic conversion was only 30% for heartwood and 60% for sapwood (Boussaid, et al. 2000). In order to improve hydrolysis, the cellulose required delignification to improve digestibility of the cellulose fraction.

Alkaline peroxide treatment of the washed solid fraction after pretreatment at 195°C, 4.5 min, 4.5% SO_2 increased hydrolytic conversion from 50% to 100% (Yang, et al. 2002). Mild alkali-oxygen treatment of washed Douglas-fir pretreated at the same conditions also increased hydrolytic conversion from 55% for untreated material to 100% conversion, but with a 10% sugar loss after delignification (Pan, et al. 2004). Fermentation of the water-soluble fraction produced from Douglas-fir pretreated at 195°C, 4.5 min, 4.5% SO_2 resulted in 92% of theoretical ethanol yield based on sugars available after pretreatment. Robinson et al. (2003) investigated the fermentability of delignified Douglas-fir hydrolysates. The enzymatic hydrolysate from alkaline-peroxide-delignified-cellulose was added to the water-soluble stream, and the ethanol production increased. However, the concentration was very low. By concentrating the hydrolysate prior to fermentation, the volume was reduced and the ethanol concentration increased (Robinson, et al. 2003). However, beyond a 2-fold concentration increase, the concentration of inhibitors was sufficiently high to prevent fermentation. Consequently, the rate of sugar consumption was extremely low for the first 24 hours. It is probable that

the difficulties in pretreating and hydrolysing Douglas-fir were due the recalcitrant physical and chemical nature of the wood.

In contrast to the difficulty in pretreating Douglas-fir, research in Sweden on Norway spruce (*Picea abies*) demonstrated that after pretreatment at 215°C, 3 min, and 2.4% SO_2, hydrolysis of the resulting material yielded 70% conversion of cellulose to glucose with no delignification required (Tengborg, et al. 2001a). Simultaneous saccharification and fermentation of the same material at 5% consistency resulted in ethanol yields of 68% of theoretical, based available sugars in the raw material (Stenberg, et al. 2000a). High consistency SSF (10% consistency) of spruce at pretreated at 215°C, 5 min, and 3% SO_2 provided an ethanol yield of 82% of theoretical based on the sugars available in the pretreated material (Rudolf, et al. 2005).

A great deal of research has gone into the technoeconomical considerations of producing ethanol from spruce. In terms of cost, SSF was found to be the most economical since it produces higher ethanol yields and has reduced capital costs (Wingren, et al. 2003). Simultaneous saccharification and fermentation at 5% consistency would result in a production cost of 0.57 USD/litre, and increasing the consistency to 8% and recycling some of the enzymes after distillation would reduce the cost to 0.42 USD/litre (Wingren, et al. 2003). Reducing the residence time to 12 hours allowed the cost to be reduced even further (Wingren, et al. 2005).

The optimization of pretreatment conditions can be complex, with a matrix of different time, temperature, and SO_2 levels, from which a relationship is derived which relates the optimum conditions for a certain outcome, such as maximal sugar recovery, high cellulose conversion during hydrolysis, or high ethanol yields after fermentation. An

alternative to using a matrix of conditions is to select from a range of conditions the one condition that provides the best results. This is useful for a variable feedstock that might change over time and not benefit from fine-tuned pretreatment conditions.

Early research focussed on optimizing for maximal recovery of glucose after pretreatment and hydrolysis (Clark and Mackie 1987; Schwald, et al. 1989), which often resulted in selection of high severity pretreatments which likely would not ferment well due to increased sugar degradation and inhibitor concentration. Recently the focus has been on selecting an optimum condition that provides the highest overall ethanol yield based on fermentable sugars in the raw material (Stenberg, et al. 1998), which requires that there is good hemicellulosic sugar recovery, minimal sugar degradation and generation of accessible cellulose during pretreatment.

Pretreatment optimization requires balancing time, temperature and acid concentration, but chip size, moisture content and wood source also play a role in determining the efficacy of the pretreatment. Chip sizes used vary considerably, from chips of 2-10 mm in size (Stenberg, et al. 2000a; Söderstrom, et al. 2002; Wingren, et al. 2005) used in much of the spruce work in Sweden, to 15x15 mm Douglas-fir chips (Cullis, et al. 2004), to 25x25 mm Radiata pine chips (Clark and Mackie 1987). Moisture content also varies, from freshly cut spruce and Radiata pine chips with over 40% moisture (Clark and Mackie 1987; Tengborg, et al. 1998), to approximately the 10% moisture content typically used for Douglas-fir (Robinson, et al. 2003). Another factor to consider is the source of the chips. The Douglas-fir chips used in studies at UBC were derived from one, 150 year old tree (Robinson, et al. 2003), while spruce used at Lund is provided by local sawmills. It has been shown that both moisture content and chip size have a significant

effect on the sugar recovery and hydrolysis yield after pretreatment (Cullis, et al. 2004), meaning that the chosen pretreatment conditions might not still be the optimum if the characteristics of the chips change. For effective optimization, the wood sample should be carefully chosen to be representative of the future feedstock supply in the area.

1.4 Mountain pine beetle-killed lodgepole pine

Previous bioconversion research at UBC utilized Douglas-fir, a major softwood species harvested in equal amounts in both the coastal and interior forests of British Columbia (COFI 1999). While Douglas-fir constitutes over a quarter of the wood harvested in coastal BC, it makes up less than 10% of the total wood harvested in the interior of the province (COFI 1999). The major softwood species in the interior is lodgepole pine, making up over a third of the timber harvested (COFI 1999). These trees are currently being devastated by the mountain pine beetle and its associated fungi, which has spread to kill millions of trees, and affect over 7% of the land area of BC (Westfall 2004). As a result, large volumes of standing dead timber are creating ghost forests across the province. This wood has unique properties, which render it unsuitable for most commercial applications. These same properties could prove beneficial for efficient bioconversion of the wood to ethanol.

1.4.1 Mountain pine beetle epidemiology

1.4.1.1 Beetle ecology

The mountain pine beetle (*Dendroctonus ponderosae*) is a normal part of the fauna of BC's pine forests. Normally endemic to BC's forests, it helps to maintain even-aged pine stands. The primary host of the beetle is Lodgepole pine (*Pinus contorta* var *latifolia*), which is found throughout Western North America (Figure 1-3). Ecologically, the beetle

is an integral part of a regeneration cycle, killing stands of older trees, which fuel forest fires that aid in the release of seeds from serotinus pine cones to grow the next generation of trees (Logan and Powell 2001).

During a beetle outbreak, trees with diameters as small as 13 cm in diameter can be attacked, provided the phloem beneath the bark is thick enough to constitute a reliable food source (Burdick, et al. 1996). It normally takes at least 40 attacks per square metre to overcome the defences of the tree (Raffa and Berryman 1983). Less than that and the tree can "pitch out" the intruders by exuding pitch through entry holes. Because of their thick phloem and weakened defences, 80-100 year old trees are the main hosts (Carroll and Safranyik 2003; Logan and Powell 2001). Once the tree's defences are exhausted, females lay eggs in vertical galleries tunnelled under the bark of the tree. The eggs mature through various stages through the winter, and emerge the following summer to attack surrounding trees (Burdick, et al. 1996).

There is some debate as to whether it is the beetle or an associated fungus that kills the tree first (Paine, et al. 1997). Beetle galleries under the bark can effectively girdle the tree by cutting off the flow of water and nutrients. In addition, the beetle carries with it spores of sapstaining fungi that germinate and kill living cells in the xylem and phloem (Safranyik, et al. 1973). The fungi, normally of the species *Ophiostoma,* (Kim, et al. 2005) are responsible for the blue stain observed in the sapwood of beetle-killed tree. There is some evidence that the rate of colonization of the fungi is insufficient to account for the foliar symptoms of tree death (Parmeter, et al. 1992), while other research has shown that without the fungus beetles are unable to successfully attack trees (Whitney 1982). It is well agreed upon that the relationship is symbiotic and mutualistic. The

fungus needs the beetle to act as a vector, and in turn benefits the beetle by lowering host defences during attack, acting as a food source for developing larvae, and creating conditions which deter other insects (Paine, et al. 1997).

After successful attack by the mountain pine beetle, trees go through green, red, and grey phases, named for the colour of the foliage. Green phase describes trees up to one year after attack. Green foliage is retained, but there is evidence of attack in the form of eggs and larvae under the bark and pitch holes on the outer bark. Red phase applies to trees approximately 2 years after attack, when the foliage turns from yellow to red. Grey phase is the entire period of time approximately 3 years after attack. During this time most of the foliage is lost, the bark peels away from trunk, and the tree finally falls to the ground where the rate of decay is much faster (Kim, et al. 2005; Koch 1996). Considering that trees can remain standing longer than 20 years after attack, there is a considerable amount of variability in the characteristics of grey phase trees.

The outbreak has so far killed over 283 million cubic metres of timber (BC Ministry of Forests 2005), and affected over seven million hectares (Westfall 2004). The area affected is shown in Figure 1-4. The annual allowable cut (AAC) in BC is only 83.8 million cubic metres (Government of BC, Forest Analysis and Inventory Branch 2006), and the volume of dead lodgepole pine far exceeds this, and exceeds the available mill capacity should the AAC be increased. The total mill capacity for BC in 2005 was only 59 million m^3, and these mills are currently running at 100% capacity (BC Ministry of Forests 2004).

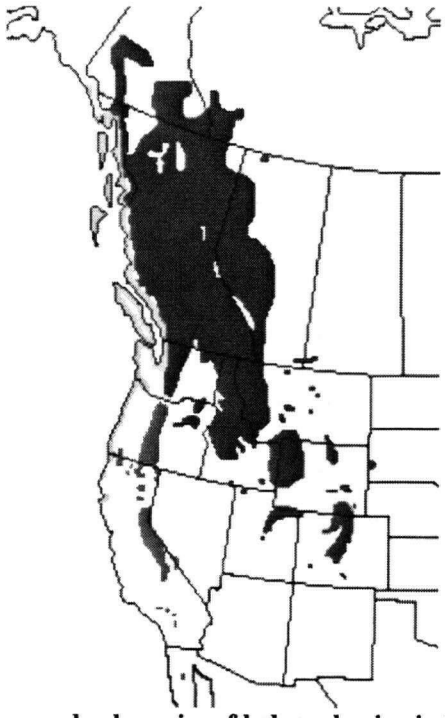

Figure 1-3. Map showing range and subspecies of lodgepole pine in North America indicating subsp. *Latifolia* in dark grey. Adapted from Critchfield and Little (1966).

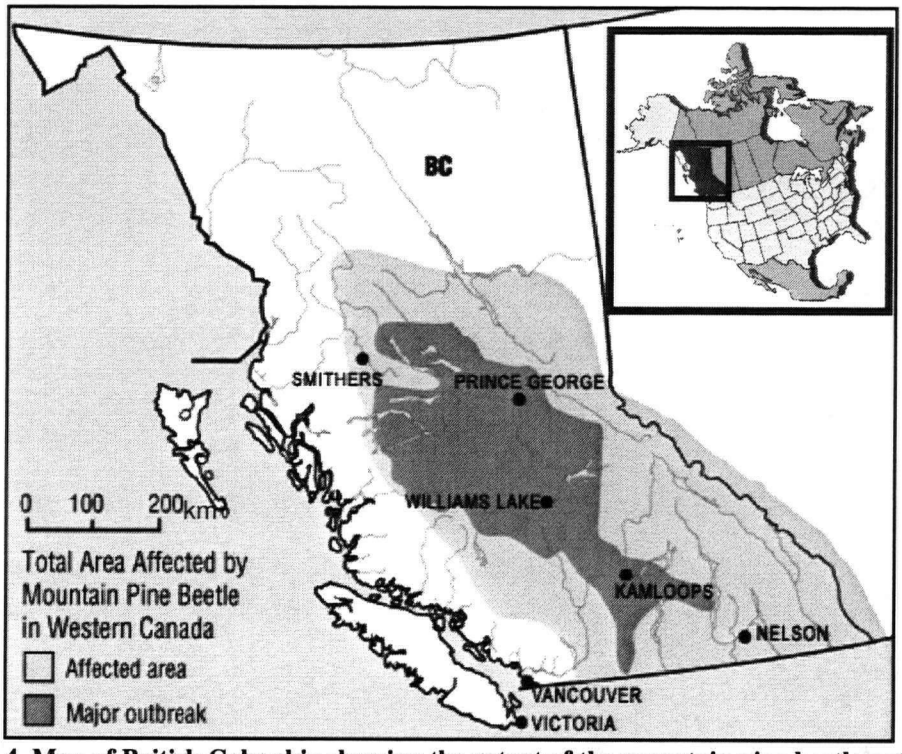

Figure 1-4. Map of British Columbia showing the extent of the mountain pine beetle outbreak. Adapted from Natural Resources Canada (2005).

1.4.1.2 Effect of climate change

One natural control mechanism for beetle outbreaks is that maturing pupae will be killed if winter temperatures drop to -40°C for a two week period in the winter (Safranyik and Linton 1998). In the past, this restricted the beetle from moving into colder areas. As mean annual temperatures have increased across the province, this has effectively increased the area in which the beetle can survive, both northward and to areas higher in altitude (Logan and Powell 2001). In favourable climates, the only barrier to the beetle's continued success is a lack of host trees. This is not likely to be a problem, as the northern boreal forests of Canada and the USA are populated by Jack pine (*Pinus banksiana*), a species that has been shown to hybridize with lodgepole pine and also provide a suitable habitat for the beetle (Cerezke 1995). If the climate in these areas becomes warm enough, there is almost no end to the number of host trees that could be available.

1.4.2 Beetle-killed lodgepole pine

1.4.2.1 Shelf life for other materials

The "shelf life" of beetle-killed wood for structural applications is variable, and depends on a number of climatic factors. Typically it can be anywhere from 1-15 years (BC Ministry of Forests 2003; Byrne, et al. 2005) before fibre loss, decay, and checking due to drying render the wood unusable for structural materials (Byrne, et al. 2005). Left standing in the forest, the infected trees become a fire hazard for surrounding communities. There are few uses for this wood, with the exception of production of pellets for burning (Stennes and McBeath 2005) or simply burning the wood itself.

Should it prove technically feasible, bioethanol production could be an ideal value-added application for this material.

1.4.2.2 Properties of healthy and beetle-killed lodgepole pine

Many differences observed between beetle-killed and healthy wood can be attributed to the dryness of the wood, particularly in trees that have been dead for a number of years. It is often difficult to determine whether observed changes are due to drying brought on by the beetle attack, or by action of the beetle and fungi themselves. Even for beetle-killed lodgepole pine, there are conflicting results from the relatively few studies that have been done in the last 50 years (Lieu, et al. 1979; Reid 1961; Thomas 1985; Woo, et al. 2005). This is likely due to a number of factors. The studies were done in different geographic areas over the wide range of lodgepole pine (Figure 1-3), on trees at different stages of attack, on both standing and downed trees, using sample sizes ranging from one to 15 trees (Lowery, et al. 1977; Woo, et al. 2005), and on trees of different ages with different ratios of sapwood to heartwood. These factors make it difficult to glean useful information from the literature regarding chemical and physical properties of beetle-killed wood. However, they highlight the importance of having a large sampling of trees from a broad area and also consistency in the age and diameter of samples.

1.4.2.2.1 Chemical composition: sugars and extractives

It is well known that sapstaining fungi consume small amounts of soluble sugars (Fleet, et al. 2001; Zabel and Morrell 1992), but lipids are a more concentrated form of energy and constitute their primary food source in infected trees (Fleet, et al. 2001). Nonetheless, this consumption of sugars could account for slightly lower carbohydrate levels observed in recently attacked trees (Woo, et al. 2005), although trees standing for longer after

attack show only marginal reductions in carbohydrates compared to healthy trees (Lieu, et al. 1979; McGovern 1951). The lignin content has sometimes been shown to decrease slightly after attack (Woo, et al. 2005). This could be attributed to the secondary colonization of the wood by decay fungi, given that sapstaining fungi are not known to degrade lignin (Scott, et al. 1996).

After beetle attack, extractive levels in wood increase for at least 2 years (Shrimpton 1973), likely due to an increase in production of defensive compounds by the tree in order to "pitch out" beetles. However, it is well known that the extractive content can be reduced by storage of logs or chips (Donetzuber and Swan 1965; Nugent and Bolker 1977), and beetle-killed pine which has been standing dead in the forest will likely be subject to the same oxidative and enzymatic processes. Oxidation occurs when oxygen reacts with double bonds in extractives and forms free radicals, which further oxidize other compounds. Enzymes act by oxidizing fatty acids and hydrolysing fats. Consequently, beetle-killed trees that have been dead for a number of years often have similar or lower extractive concentrations compared to healthy trees (Lieu, et al. 1979). In addition, most sapstaining fungi consume extractive compounds, particularly lipids (Fleet, et al. 2001).

1.4.2.2.2 Physical differences: moisture content, permeability, and fibre quality

It is well established that the moisture content of beetle-infested lodgepole pine declines sharply after attack (Reid 1961; Thomas 1985). Typically the moisture of the sapwood is 85-165% of oven dry weight (Reid 1961). Within as little as 8 months after attack, the moisture can drop to 25% or as low as 16% after one year (Reid 1961; Woo, et al. 2005). This is below the fibre saturation point of 30% of oven dry weight, beyond which the

structure of the wood begins to collapse as it dries further (Sjöström 1993). This leads to checking in the wood to relieve the drying stress. The drop in moisture is likely due to the blockage of xylem tracheids by fungal hyphae, release of gas bubbles into tracheids and/or production of substances that block pit openings (Nebeker, et al. 1993).

Drying of the wood can reduce its permeability by causing aspiration of the bordered pits that link tracheids (Comstock and Côté 1968; Siau 1984). Pit aspiration can also be caused when the pit membrane becomes encrusted with extractives (Siau 1984), as often occurs during fungal infection (Nebeker, et al. 1993). A reduction in permeability has implications for pretreatment of this material since uptake of catalytic substances will be reduced. Contrary to this, some authors have reported increased permeability of beetle-killed wood (Woo, et al. 2005; Zabel and Morrell 1992), hypothesizing that the fungi remove pit membranes. However, these studies were carried out on recently killed wood, and further drying of grey-phase trees may alter the permeability.

It has been well established that dry wood leads to an increase in fines during chipping. Thomas (1985) showed that chipping of beetle-killed lodgepole pine generated more fines with increased time after attack. This has negative ramifications for the pulping industry, as it reduces the uniformity of chip sizes before screening, and reduces the amount of acceptable chips after screening. For the purposes of bioconversion, a reduced particle size might be beneficial, as it increases the available surface area for pretreatment and subsequent hydrolysis (Mansfield, et al. 1999).

1.5 Research objectives

This thesis aimed to explore the bioconversion behaviour of SO_2-catalyzed steam pretreated beetle-killed lodgepole pine and compare it to a comparable sampling of

healthy wood. Previous work on Radiata pine (Clark and Mackie 1987) suggested that lodgepole pine could potentially be hydrolysed efficiently with no delignification. If this was the case, lodgepole pine might exhibit bioconversion characteristics more similar to spruce than to Douglas-fir, resulting in promising ethanol yields.

1.5.1 Validation of the steam explosion process at UBC

Past work using steam pretreated material produced at UBC (Douglas-fir) and Lund University in Sweden (primarily spruce) resulted in quite different outcomes. The need to delignify was apparent when using Douglas-fir (Wu, et al. 1999), while work with spruce and pine (Clark and Mackie 1987; Tengborg, et al. 2001a) showed high hydrolytic conversion could be achieved without the need to delignify. This is most likely a result of the fact that the feedstock used at UBC, Douglas-fir, is much more recalcitrant than the spruce used at Lund. However, in order to confirm that our steam gun is comparable to the one used at Lund, we pretreated spruce using our equipment and compared it to the same raw material pretreated at Lund. The idea was that, if the results showed that the spruce pretreated at UBC and Lund were indeed comparable, this would indicate that pretreatment of other materials using the UBC gun would yield reproducible substrates which might be studied elsewhere, and furthermore, that there is a fundamental difference between the bioconversion properties of spruce and Douglas-fir regardless of the equipment used.

1.5.2 Optimization of pretreatment conditions for beetle-killed lodgepole pine

In order to find the optimum set of SO_2-catalyzed steam pretreatment conditions for beetle-killed lodgepole pine, we selected 7 different conditions based on known optima

for other feedstocks. Based on sugar recovery and hydrolysis results from these substrates, we chose three conditions; low, medium and high severity. These provided high sugar recovery (low severity), high hydrolytic conversion (high severity) or both (medium severity). These three substrates subsequently underwent fermentation, separate fermentation and hydrolysis (SHF), and simultaneous saccharification and fermentation (SSF). The optimum severity was chosen based on the overall ethanol yield based on available sugars in the raw material.

1.5.3 Robustness of chosen pretreatment

Future feedstock supplies in BC are likely to be a mixture of healthy and beetle-killed lodgepole pine, with the proportions of each changing over time and space. The optimum conditions selected for beetle-killed lodgepole pine should be ones that are robust enough to give good results on a wide range of wood types. By applying these conditions to healthy and a 50/50 mixture of healthy/beetle-killed, we evaluated whether there were comparable sugar recovery and ethanol yields compared to beetle-killed material.

1.5.4 Comparison of healthy to beetle-killed lodgepole pine

To evaluate whether there were differences in the bioconversion behaviour of healthy and beetle-killed pine, both were compared at low, medium, and high severities as defined in the optimization section. Differences in hydrolytic performance and ethanol yields after SSF and SHF were observed. Some differences between healthy and beetle-killed wood that may have led to the observed differences in bioconversion were hypothesized.

2 MATERIALS AND METHODS

2.1 Experimental conditions

Experiments were conducted once due to time and equipment constraints, with all samples in duplicate unless otherwise indicated. As a result, all error bars shown in figures are based on the deviation of the duplicates from the mean.

The solid particles produced after pretreatment were quite varied in size due to the large variation in size of the wood chips in the starting material (Figure 2-6). The insoluble fraction after pretreatment was not screened in any way to remove oversized chunks. This resulted in some variability between duplicates in any given experiment, producing a larger range of results. This error was usually larger at the beginning of the experiment, while by the end any larger particles which were slower to hydrolyse would have been hydrolysed to their full extent and subsequently, the differences between duplicates was reduced.

Ethanol yields were calculated as the percent of theoretical ethanol yield given that *S. cerevisiae* produces 0.51 g ethanol per g hexose under ideal conditions (Hahn-Hägerdal, et al. 1994). Experimental yields were calculated based on the fermentable sugars available in the pretreated materials at the start of the experiment. Overall yields were calculated using initial concentration of hexoses in the starting raw material before pretreatment and thus reflect any loss of sugar during pretreatment.

2.2 Comparison of spruce pretreated at Lund and at UBC

Past differences in cellulose hydrolysability observed between spruce pretreated at Lund University and Douglas-fir pretreated at UBC prompted a comparison of the efficacy of the two processes on the same substrate, spruce (Figure 2-1).

2.2.1 Raw and pretreated material

Raw, chipped spruce was obtained from Lund University, Sweden. The chip size was approximately 10 x 5 x 5 mm with 59% moisture content (Figure 2-2), and the material was stored at 4°C after arrival. The composition of the chips was determined at UBC, and it was close to the literature values for a similar sample of spruce (Table 2-1).

Lund also supplied pretreated material (conditions described below) that was shipped at 4°C and frozen after arrival. The material arrived as a slurry of water-soluble and insoluble fractions with 89% moisture content. The solids were separated from the liquid stream using vacuum filtration and washed with a volume of water 20 times the dry weight.

Table 2-1. Composition of raw spruce before pretreatment. Values in parentheses represent the deviation from the mean.

	G/100g total dry weight	
	Spruce	Reference[a]
Arabinose	0.9 (0.0)	0.9
Galactose	1.7 (0.0)	2.4
Glucose	46.3 (0.1)	49.7
Mannose	12.5 (0.1)	14.8
Xylose	5.4 (0.1)	5.1
Acid insoluble lignin (AIL)	28.2 (0.6)	27.7
Acid soluble lignin (ASL)	0.5 (0.0)	-
Total	95.7 (0.9)	100.6

[a]From (Stenberg, et al. 2000a).

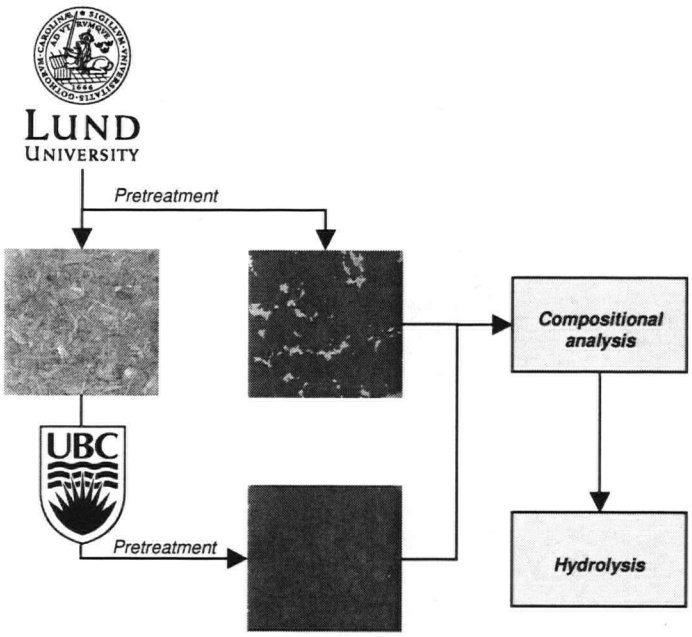

Figure 2-1. Comparison of spruce pretreated at Lund University to the same raw material pretreated at UBC.

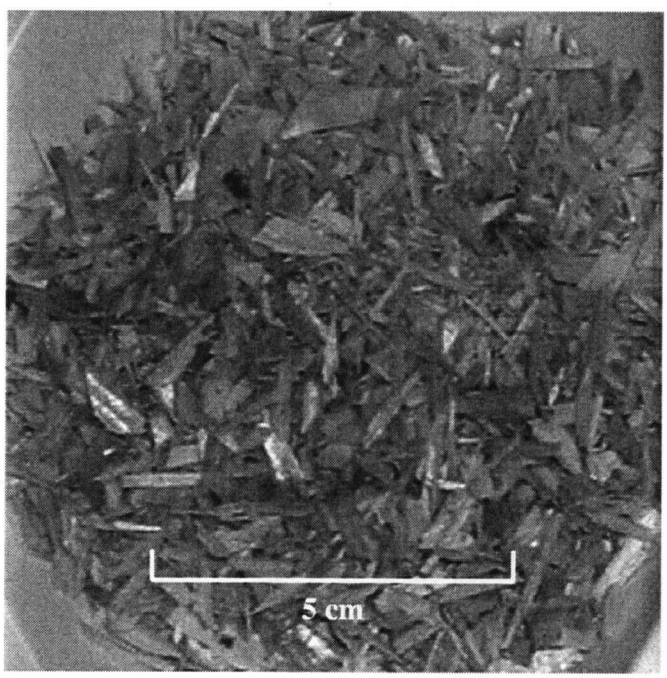

Figure 2-2. Chipped spruce sent from Lund University.

2.2.2 Pretreatment of raw material at UBC

The steam-exploded spruce that was sent was pretreated using conditions commonly used by researchers at Lund: 215°C, 5 min, 3% m.c. SO_2. The only difference in their procedure from that used at UBC is that the chips are typically freshly chipped, and therefore at a high moisture content (~60%). SO_2 (Praxair Canada) was added according to the moisture content in the wood, not the dry weight. For 300 g dry weight (d.w.) of chips at 59% moisture, (732 g wet weight), 13 g of SO_2 was added. This was comparable to adding 4% d.w. SO_2 to the same dry weight of chips at any moisture content. For the comparison of spruce pretreated at Lund and UBC, we used the Lund method for calculating the amount of SO_2 added.

After impregnating the chips with SO_2 overnight, they were re-weighed in the morning, and found to have retained 88% of the SO_2 added. The chips were divided into 50 g d.w. portions, which were treated separately in the steam gun for 5 minutes at 215°C. After all 6 samples had been discharged to the collecting vessel, it was opened and the pretreated material removed. The water-insoluble fraction (WIF) was separated from the water-soluble (WSF) using vacuum filtration and the solids washed with a volume of water twenty times the dry weight. Both fractions were stored at 4°C.

2.2.3 Compositional analysis and hydrolysis

The composition of the two pretreated substrates was analysed as detailed below in section 2.5.1, and the conditions for hydrolysis are outlined in section 2.6.1.

2.3 Lodgepole pine pretreatment

Beetle-killed and healthy lodgepole pine were obtained in the form of bolts of wood. These were debarked, chipped, screened and pretreated. The resulting substrates underwent hydrolysis, fermentation, separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) (Figure 2-3).

2.3.1 Raw material

The beetle-killed lodgepole pine used for this study was carefully selected in order to represent the wide variation in characteristics that occurs over an area as large as British Columbia. Similarly, healthy wood was selected which had as many characteristics as possible in common with the beetle-killed wood, primarily age and diameter.

2.3.1.1 Tree characteristics

Beetle-killed lodgepole pine was kindly provided by Dr. Colette Breuil at UBC in the form of fourteen bolts of wood from upper (cut 3.5 m from the ground) and lower (cut 0.3 m from the ground) sections of seven grey-phase trees located in central British Columbia. The trees had been infected and killed 3 years prior to harvest. Healthy lodgepole pine was made up of bolts of wood cut at a height of 0.3 m from the ground from four non beetle-attacked lodgepole pines in south-central BC. They were generously provided by Ken Ewanick at Tolko Industries Ltd. in Vernon, BC. Individual tree locations are shown in Figure 2-4. All of the bolts of wood were inspected visually to make sure there were no discernible signs of rot.

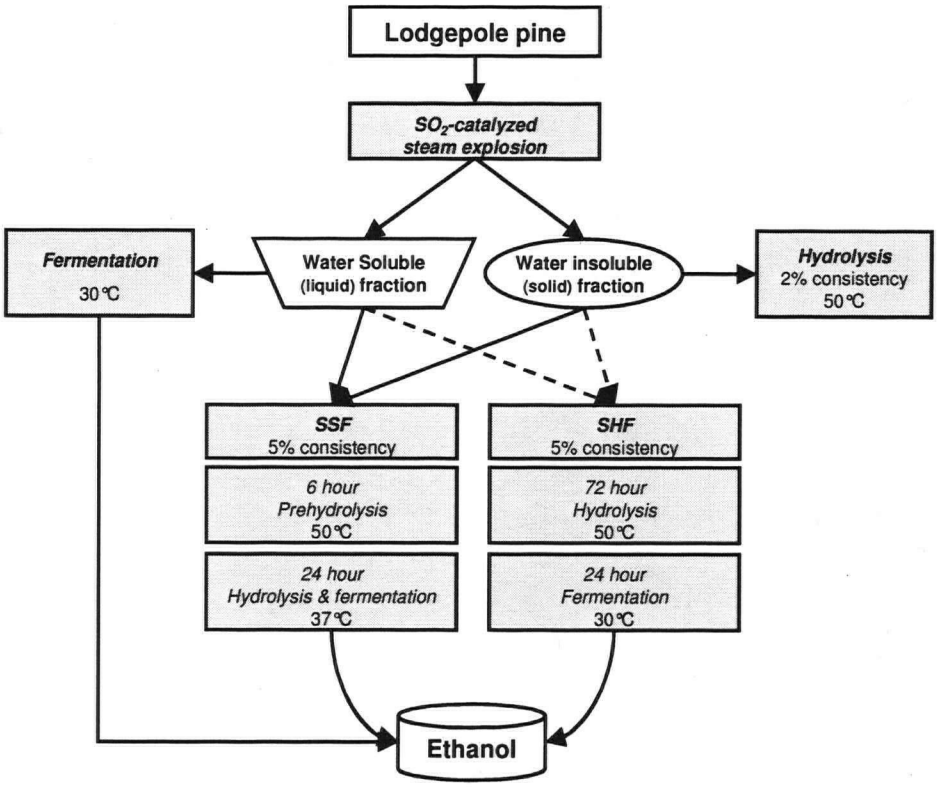

Figure 2-3. Processes utilized during bioconversion of lodgepole pine to ethanol.

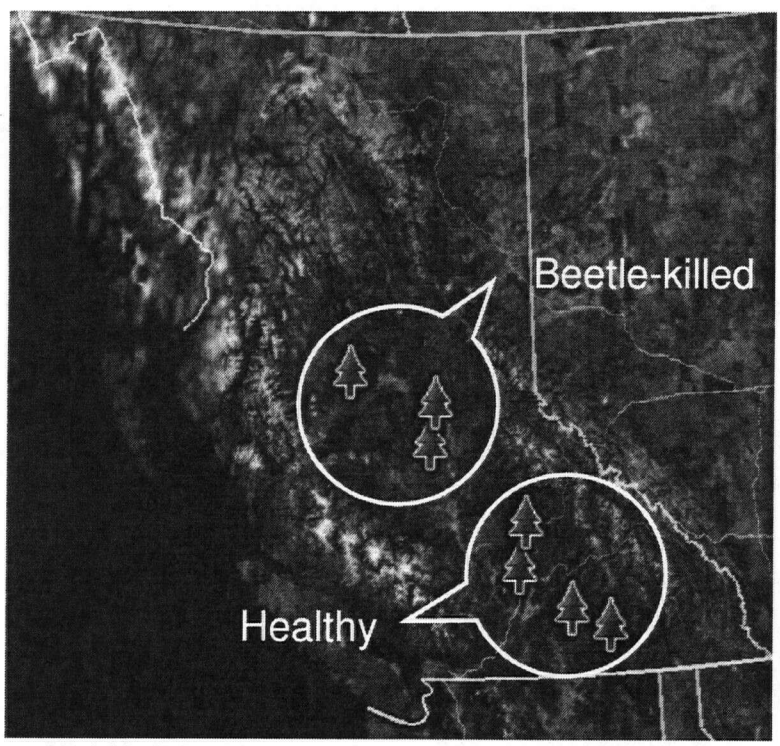

Figure 2-4. Map of British Columbia showing the locations of healthy and beetle-killed lodgepole pine trees used in this study.

The average age of the beetle-killed and healthy trees was 99-101 years, although there was a 20-25% standard deviation for each (Table 2-2). The average diameter of the healthy trees was slightly higher than the beetle-killed, though the standard deviation was 17-23% for both. The healthy trees were harvested a year later than the beetle killed, and the bolts were debarked and chipped in May 2005 (healthy) and December 2005 (beetle-killed). The bolts of each type are pictured in Figure 2-5.

Table 2-2. Characteristics of lodgepole pine trees used for raw material. Values in parentheses represent the standard deviation.

	Beetle-killed	Healthy
Average age (years)	99 (25)	101 (20)
Average diameter (cm)	24 (4)	26 (6)
Harvest date	Fall 2004	Fall 2005
Number of trees in sample	7	4

2.3.1.2 Composition

Both sets of samples were debarked, split, chipped and screened to 20 x 20 x 5 mm (Figure 2-6). While the beetle-killed wood was dry on arrival, the healthy wood had been freshly cut. It was debarked immediately, allowed to partially air dry at room temperature, and then split. The split pieces were further air dried at room temperature prior to chipping, after which the final moisture content of the wood was measured. The chips of each type (beetle-killed and healthy) were combined and thoroughly mixed, and the final moisture content was 12% for beetle-killed and 11% for healthy. The third feedstock, mixed, was made by mixing equal parts beetle-killed and healthy chips. The composition of the raw material (Table 2-3) was determined according to the method in section 2.5.1.

Table 2-3. Composition of raw lodgepole pine before pretreatment. Values in parentheses represent the deviation from the mean.

	g/100g total dry weight	
	Beetle-killed	Healthy
Arabinose	1.9 (0.0)	2.1 (0.0)
Galactose	3.0 (0.0)	3.3 (0.1)
Glucose	46.6 (0.4)	49.9 (0.1)
Mannose	12.4 (0.1)	12.8 (0.3)
Xylose	6.6 (0.1)	6.9 (0.1)
Acid insoluble lignin (AIL)	28.5 (0.3)	26.2 (0.2)
Acid soluble lignin (ASL)	0.6 (0.0)	0.4 (0.0)
Extractives	3.6 (0.4)	3.4 (0.5)
Total	102.8 (1.3)	104.7 (1.3)

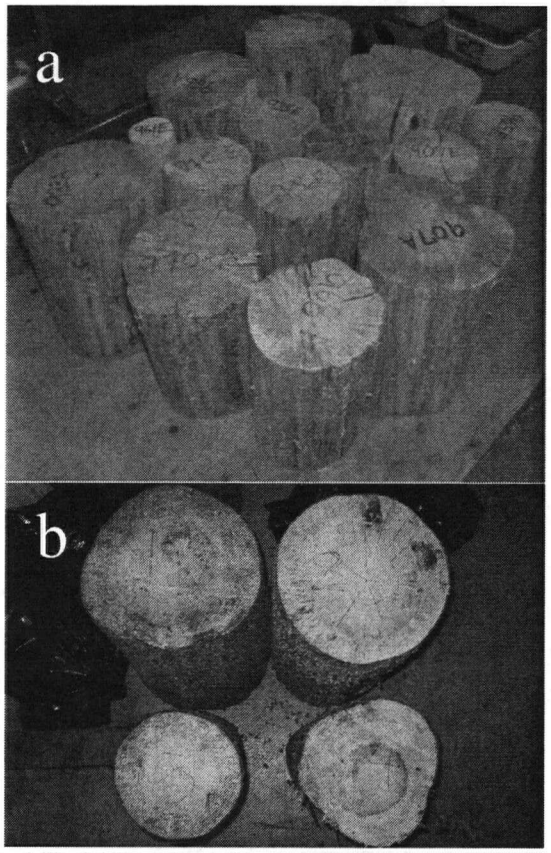

Figure 2-5. Bolts of beetle-killed (a) and healthy (b) lodgepole pine.

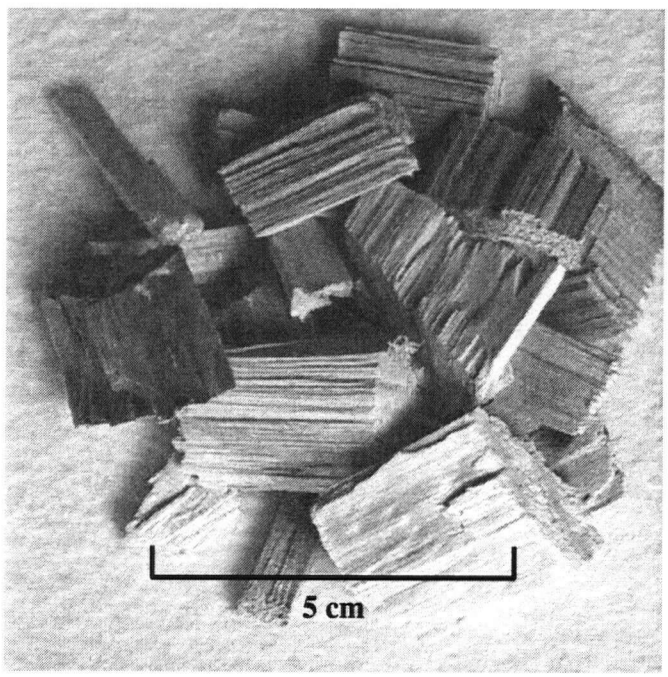

Figure 2-6. Chipped and screened lodgepole pine.

2.3.2 Pretreatment

A complete response surface methodology study to determine the optimum pretreatment was not done because of the large amount of variability inherent between different trees, particularly trees at different stages of attack and from an area as large as British Columbia. The precise optimum for one sample of wood may not be the optimum for another sample. Thus, a preliminary range of 7 conditions were chosen, with the least severe being the conditions used to pretreat Douglas-fir and the most severe being the optimum conditions used for Spruce at Lund University. The other 5 conditions were chosen to fall in between (Table 2-4).

Table 2-4. Conditions and corresponding severities used for optimization of pretreatment of beetle-killed lodgepole pine and resulting hexose recoveries.

Severity Code	Temp (°C)	Time (min)	SO_2 (% d.w.)	Severity ($logR_o$)
S1	195	4.5	4.5	3.45
S2	**195**	**7.5**	**4**	**3.67**
S3	**200**	**5**	**4**	**3.64**
S4	205	5	2	3.79
S5	200	10	2	3.94
S6	210	5	2	3.94
S7	**215**	**5**	**4.5**	**4.09**

Prior to steam explosion, wood chips were impregnated with sulphur dioxide in the amount shown in Table 2-4 by adding gaseous SO_2 (Praxair Canada) to plastic bags containing 300 g d.w. of wood chips. The bags were weighed and left at room temperature overnight. In the morning, any excess gas was released and the bags were reweighed to measure how much acid had been absorbed. An average of 78% of the SO_2 was absorbed by the beetle-killed wood, and 95% by the healthy. The impregnated chips were added to the reactor of a 2-L StakeTech II steam gun (Stake Technology, Norval

Ontario) in 50 g d.w. portions and treated at the specified temperature and time shown in Table 2-4. After 300 g d.w. had been discharged to the collecting vessel, the slurry was removed and stored at 4°C. After the bulk of the material had been removed, the vessel was rinsed with tap water and the liquid collected separately and analysed for sugars in order to improve the closure of the mass balance.

The water-soluble fraction (WSF) was separated from the water-insoluble fraction (WIF) by vacuum filtration. The WIF was then washed with a volume of water twenty times the dry weight. Monomeric and oligomeric sugar concentrations were determined for the WIF wash liquid, steam gun wash liquid, WSF, and WIF to calculate the sugar recovery.

2.4 Yeast preparation

The Tembec T1 strain of *Saccharomyces cerevisiae* (provided by Tembec Limited, Témiscaming, Québec, Canada) was maintained on plates of solid media containing 10 g/l yeast extract (Fisher), 20 g/l peptone (Fisher), 20 g/l glucose (Sigma) and 18 g/l agar at 4°C. This organism has been adapted to spent sulphite liquor and exclusively ferments hexoses.

Cells were grown by transferring a colony from a plate to a 1-L flask containing 400 ml liquid media (10 g/l yeast extract, 10 g/l peptone, and 10 g/l glucose) in an orbital shaker at 30°C and 150 rpm. The medium was replaced after 24 hours and the cells harvested after 48 hours by centrifugation at 3000 rpm for 10 minutes. The supernatant was removed and the cells washed and centrifuged the same way 3 times with sterile distilled water. The concentration of cells was adjusted to 100 g/l with water so that 2.5 ml added to 47.5 ml of solution would give a yeast concentration of 5 g/l. The yeast concentration

was determined by relating the optical density at 600 nm to a standard curve where OD_{600} = 1.527 [yeast] (g/l).

2.5 Enzymatic hydrolysis and fermentation

All experiments were carried out in 125 ml Erlenmeyer flasks in duplicate. The total solution volume was 50 ml in all cases except for simultaneous hydrolysis and fermentation (SHF). Antibiotics were used only in the 2% consistency hydrolysis and never in fermentation experiments.

2.5.1 Hydrolysis

Washed solids were enzymatically hydrolysed at 2% (w/v) consistency in acetate buffer (Fisher) (50mM, pH 4.8) at 50°C and 150 rpm. Enzymes were added in the form of cellulase at 20 FPU/g cellulose (Spezyme; Genencor: Palo Alto, CA, USA) and β-glucosidase at 10 CBU/g cellulose (Novozymes 188; Novozymes: Franklinton, NC, USA). 40 μg/ml tetracycline (Sigma) and 30 μg/ml cycloheximide (Sigma) were added to inhibit bacterial and fungal contamination. 400 μl samples were taken periodically over 48 hours, boiled for 5 minutes and stored at -20°C.

2.5.2 Fermentation

47.5 ml of undiluted WSF were adjusted to pH 6 with 50% NaOH (Fisher) and 2.5 ml yeast was added for a final concentration of 5 g/l. Flasks were incubated in an orbital shaker at 30°C and 150 rpm and 400 μl samples taken periodically over 48 hours, centrifuged at 10,000 rpm for 5 minutes and the supernatant stored at -20°C.

2.5.3 Separate hydrolysis and fermentation (SHF)

Washed solids were diluted to 5% (w/v) consistency with the WSF and the solution adjusted to pH 4.8 with 50% NaOH. The total volume used was 70 ml in order to allow enough liquid to be recovered for the fermentation step. Cellulase at 40 FPU/g cellulose (Spezyme) and β-glucosidase at 20 CBU/g cellulose (Novozymes 188) were added and the flasks incubated in an orbital shaker at 50°C and 150 rpm for 72 hours. 400 µl samples were taken periodically over 72 hours, boiled for 5 minutes and stored at -20°C.

After completion of the hydrolysis step, the reaction mixture was boiled for 5 minutes, and then centrifuged at 10,000 rpm for 10 minutes to separate the hydrolysate, which was removed and measured into clean flasks. The pH was adjusted to 6 with 50% NaOH and 5 g/l yeast added. Total volume for the fermentation was 40 ml. The flasks were incubated in an orbital shaker at 30°C and 150 rpm for a further 48 hours. 400 µl samples were taken periodically, centrifuged at 10,000 rpm for 5 minutes and the supernatant stored at -20°C.

2.5.4 Simultaneous saccharification and fermentation (SSF)

The SSF protocol originally used on delignified Douglas-fir and agricultural residues in the Forest Products Biotechnology group at UBC did not produce satisfactory ethanol yields from pretreated lodgepole pine. The method was adapted through the following experiments detailed in section 2.5.4.1, and the final modified procedure presented in section 2.5.4.2.

2.5.4.1 SSF development

2.5.4.1.1 Original SSF procedure

Washed solids were diluted to 5% (w/v) consistency with the WSF and the solution adjusted to pH 5 with 50% NaOH. 5 g/l yeast, cellulase at 40 FPU/g cellulose (Spezyme) and β-glucosidase at 20 CBU/g cellulose (Novozymes 188) were added and the flasks incubated in an orbital shaker at 37°C and 150 rpm. 400 µl samples were taken periodically, centrifuged at 10,000 rpm for 5 minutes and the supernatant stored at -20°C.

2.5.4.1.2 Effect of pH and yeast concentration

The original SSF protocol was followed, with the following changes: the pH was monitored for two of the samples by measuring pH at every timepoint. One sample was maintained at pH 5 with additions of NaOH as required, while the other one was not adjusted for a specific pH. The third sample was given an extra "dose" of yeast at 10 hours, for a total of 10 g/l yeast per flask.

2.5.4.1.3 Effect of nutrient addition and prehydrolysis

The original SSF protocol was followed, with the following changes: nutrients consisting of 0.5 g/l $(NH_4)_2HPO_4$, 0.025 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l yeast extract and 1 g/l peptone were added to half the flasks. The other flasks were given the same amount of water. Sets of flasks with and without nutrients were subjected to prehydrolysis for 6 or 12 hours at 50°C and 150 rpm prior to adding the yeast. This time was not included in the total SSF time. In addition, the control samples did not undergo prehydrolysis. After prehydrolysis, all flasks underwent SSF for 24 hours.

2.5.4.1.4 Effect of second addition of yeast

The original SSF protocol was followed, with the following changes: nutrients added to all of the samples in the concentrations detailed in the previous section. Prehydrolysis was carried out for 6, 12, or 24 hours as detailed above, except for a control sample which did not undergo prehydrolysis. All samples were given the same amount of yeast (5 g/l) at the start of SSF, and after 11 hours half of the samples were given another "dose" of yeast of for a total of 10 g/l yeast per flask.

2.5.4.2 Adapted SSF procedure

Washed solids were diluted to 5% (w/v) consistency with the WSF and the solution adjusted to pH 5 with 50% NaOH. Nutrients (0.5 g/l $(NH_4)_2HPO_4$, 0.025 g/l $MgSO_4 \cdot 7H_2O$, 1 g/l yeast extract and 1 g/l peptone) were added to each flask. Cellulase at 40 FPU/g cellulose (Spezyme) and β-glucosidase at 20 CBU/g cellulose (Novozymes 188) were added and the flasks incubated in an orbital shaker at 50°C and 150 rpm for 6 hours. After this prehydrolysis, 5 g/l yeast was added to each flask and the temperature reduced to 37°C. 400 µl samples were taken periodically, centrifuged at 10,000 rpm for 5 minutes and the supernatant stored at -20°C.

2.6 Analysis of sugars, extractives and ethanol

2.6.1 Carbohydrates and lignin

Monosaccharides were measured on a Dionex (Sunnyvale, CA) HPLC (ICS-2500) equipped with an AS50 autosampler, ED50 electrochemical detector, GP50 gradient pump and anion exchange column (Dionex, CarboPac PA1). Deionised water at 1 ml/min was used as an eluent, and postcolumn addition of 0.2 M NaOH maintained baseline stability and detector sensitivity. After each analysis, the column was reconditioned with

1 M NaOH. 20 µl of each sample were injected after filtration through a 0.45 µm syringe filter (Chromatographic specialities, Brockville, Canada). Standards were prepared containing sufficient arabinose, galactose, glucose, xylose and mannose (all Sigma) to encompass the same range of concentrations as the samples. 0.2 g/l fucose (Sigma) was added to all samples and standards as an internal standard.

Posthydrolysis analysis of all liquid samples allowed quantification of the amount of oligomeric sugars present. 0.7 ml of 70% H_2SO_4 was added to 15 ml of the liquid sample and the volume made up to 20 ml with water. Samples were autoclaved at 121°C for one hour and analysed by HPLC as described above.

Solid samples were analyzed for insoluble (Klason) lignin and sugars using the modified Tappi T-222 om-88 method (TAPPI 1998). Briefly, the raw wood or pretreated material was air-dried and ground to pass through a 40-mesh screen. 0.2 g of the ground sample was mixed with 3 ml of 72% sulphuric acid (Fisher) and incubated at 20°C for 2 hours with stirring of the solution every 10 minutes. Following the acid hydrolysis, the reaction mixture was diluted with 112 ml of deionised water for a total volume of 115 ml and 4% acid concentration. The solution was transferred to a serum bottle and autoclaved at 121°C for one hour. After cooling to room temperature, mixture was filtered through a medium-coarseness sintered glass crucible. The filtrate was transferred to a falcon tube, and the sugar concentrations measured by HPLC. Soluble lignin in the filtrate was determined by absorbance at 205 nm (Dence 1992). The insoluble Klason lignin remaining in the crucible was washed with deionised water and the crucible dried overnight at 100°C. The amount of Klason lignin was then determined gravimetrically

after weighing the oven-dried crucible, taking into account the initial moisture content of the sample.

2.6.2 Extractives

Acetone-soluble extractives were determined in duplicate using modified Tappi T204 om-88 (TAPPI 1987). Briefly, the sample was air-dried and ground to pass through a 40-mesh filter. 10 g d.w. of this material was weighed into a coarse sintered glass crucible, which was placed inside a Soxhlet apparatus. The Soxhlet apparatus was connected to a cold water condenser and then connected to a 250 ml round-bottomed flask containing 100 ml acetone. The acetone was refluxed with 6 cycles per hour for a minimum of 8 hours. Following this, the crucible was removed and dried at 100°C for at least 24 hours. The extractive content was determined gravimetrically, taking into account the initial moisture content of the sample. No analysis was done on the extracted material in the acetone fraction.

2.6.3 Ethanol

Ethanol was analysed using gas chromatography on a Hewlett Packard 5890 GC equipped with a Stabilwax-DA column (30 m, 0.35 mm ID) and helium carrier gas (20 ml/min). Injection and FID temperatures were 90 and 250°C respectively. The oven was heated at 45°C for 6 minutes and increased to 230°C at a rate of 20°C/min, then held at 230°C for 10 minutes. Standards were prepared using 99% ethanol (Riedel de Haen) at a range of concentrations. 0.3 g/l butanol (Fisher) was added to all samples and standards as an internal standard.

2.6.4 HMF and furfural

Furfural and 5-hydrolymethyl furfural (HMF) were analyzed on a Summit HPLC (Dionex) equipped with a Lichrospher RP18 reversed phase column (Varian Instruments, Walnut Creek, CA) and a PDA-100 detector set at 280 nm. Standards were prepared using HMF (Sigma) and furfural (Sigma) diluted in water. Catechol (BDH) was added as an internal standard to all samples and standards prior to their filtration through a 0.45 μm syringe filter (Chromatographic specialities, Brockville, Canada). The injection volume was 25 μl. The column was heated to 60°C for the duration of the analysis and eluted at a flow rate of 0.5 ml/min with a ternary gradient of 7.4 mM phosphoric acid (Fisher) (eluent A), acetonitrile (Fisher) (eluent B), and a 4:3:3 v/v solution of 7.4 mM phosphoric acid, methanol (Fisher), and acetonitrile (eluent C). The elution profile consisted of a 20 minute gradient of 95% eluent A and 5% eluent C transitioning to 50% eluent A and 50% eluent C. This was followed by 4 minutes of gradient to 100% eluent C, a 1 minute hold, then a 1 minute transition to 100% eluent B. After holding for 1 minute, there was a 1 minute transition from 100% eluent B back to 95% eluent A and 5% eluent C, followed by 10 minutes of re-equilibration to end the run for a total time of 38 minutes.

3 RESULTS AND DISCUSSION

3.1 Overview

Prior to any investigations using steam exploded material, the steam explosion process used at UBC was compared to the one used at Lund by comparing substrates produced from both steam guns (Section 3.2). Past results had shown that Douglas-fir pretreated at UBC was poorly hydrolysed compared to spruce pretreated at Lund. Comparing the same substrate pretreated at each location would help to determine whether the observed differences were due to the wood species or to the process.

Following this, we investigated how beetle-killed lodgepole pine performed as a feedstock during bioconversion. First, the steam pretreatment conditions were optimized for maximum ethanol yield after SSF by selecting the "optimum" condition from a range of conditions (Section 3.3). Then, the "optimum" pretreatment condition was applied to beetle-killed, healthy, and mixed beetle-killed/healthy pine in order to see whether the pretreatment was sufficiently robust to provide comparable ethanol yields on other feedstocks (Section 3.4). Finally, healthy wood was pretreated at three different severity conditions and compared to the corresponding beetle-killed substrates in order to see whether there were consistent differences between the two substrates (Section 3.5).

After evaluating the ethanol yields obtained at different pretreatment severities from both beetle-killed and healthy wood, two questions could be answered. Firstly, whether lodgepole pine was a suitable candidate for bioconversion compared to other softwoods, and secondly, whether there was a difference between ethanol yields obtained from healthy and beetle-killed wood.

3.2 Validation of UBC steam explosion process

3.2.1 Rationale

Before embarking on a research plan involving bioconversion of steam-pretreated softwood, it seemed prudent to ensure that our steam gun was producing substrates comparable to those produced by similar guns elsewhere. This was brought on by the fact that Douglas-fir treated at UBC provided only 24% conversion of cellulose to glucose at conditions which provided 77% sugar recovery (Wu, et al. 1999). Increasing the severity increased the hydrolysability of the cellulose to 98%, but sugar recovery decreased to 43% and the water-soluble fraction did not ferment due to high concentrations of inhibitors (Boussaid, et al. 1999; Wu, et al. 1999). In contrast, work carried out at Lund University showed that hydrolytic conversions of 70% could be achieved from pretreated spruce, and the corresponding water-soluble-fractions generated were readily fermentable (Stenberg, et al. 1998; Tengborg, et al. 1998). It was suggested that the major differences observed between spruce and Douglas-fir were most likely due to chemical and structural differences between the two wood species, as it is well know that Douglas-fir is harder and denser than spruce (Green, et al. 1999). However, there was a possibility that the differences were due to the pretreatment equipment used, and this possibility had to be assessed before any further steam explosion work was carried out done at UBC.

In cooperation with researchers at Lund University in Sweden, we compared spruce pretreated at their facility and at ours. They supplied us with both steam-exploded and raw spruce, and we pretreated the raw material in our gun using the same conditions used on the Lund-treated material. The composition and hydrolysability of both substrates were compared. Since poor hydrolysability was the main problem with pretreated

Douglas-fir, the fermentability of the soluble, hemicellulose-rich fractions was not compared.

3.2.2 Composition of pretreated spruce

The composition of the washed, water-insoluble fraction was similar for both substrates, with the Lund-treated material containing 7% less glucose and 9% more insoluble lignin than substrate pretreated at UBC (Table 3-1). Compared to published values for pretreated spruce, the glucose content is 2-9% lower and the lignin content 11-23% higher. These differences are likely a result of slightly different raw feedstock characteristics and pretreatment conditions of the reference substrate. Researchers at Lund add SO_2 based on the moisture content of the raw wood, so a drier wood would result in the introduction of less SO_2. Indeed, the moisture content of the spruce used for the reference values was 48% (Stenberg, et al. 2000a), compared to the 59% moisture content of the spruce used for the work presented here, resulting in the addition of 25% less SO_2 during pretreatment of the reference material.

Table 3-1. Composition of spruce pretreated at Lund and UBC at 215°C, 4.5 min, 3% m.c. SO_2. Values in parentheses indicate deviation from the mean.

	g/100g dry weight		
	Lund-pretreated	UBC-pretreated	Reference[a]
Arabinose	0.0 (0.0)	0.0 (0.0)	-
Galactose	0.1 (0.0)	0.1 (0.0)	-
Glucose	51.9 (0.3)	56.1 (0.9)	57
Mannose	0.3 (0.1)	0.4 (0.2)	-
Xylose	0.1 (0.1)	0.4 (0.1)	-
Acid insoluble lignin (AIL)	47.9 (0.7)	43.4 (0.8)	39
Acid soluble lignin (ASL)	1.3 (0.0)	0.8 (0.0)	-
Total	101.6 (1.2)	101.2 (2.0)	96

[a]**From (Stenberg, et al. 2000a). Material was pretreated at 215°C, 5 min, 2.8% m.c. SO_2.**

3.2.3 Hydrolysis of pretreated spruce

The hydrolysis results of the two substrates were also similar, with no significant difference between hydrolytic conversions of 79 and 76% of cellulose to glucose for Lund and UBC-pretreated spruce respectively (Figure 3-1). Previous work carried out at Lund using the same substrate in a 1-L stirred reactor at 40°C and similar enzyme loading showed 62% conversion (Tengborg, et al. 2001a), likely due to the reduced temperature (we used 50°C) and slightly different substrate characteristics.

3.2.4 Conclusion

Since the composition and hydrolytic performance of the two substrates were comparable, it was reasonable to assume that the two steam guns were producing similar substrates. This suggested that the differences previously observed between pretreated spruce and Douglas-fir were primarily due to the nature of the wood, and not the pretreatment. In addition, the successful pretreatment of spruce at UBC meant that pretreated lodgepole pine would likely also provide high hydrolytic conversion without the need for delignification, especially since other species of pine have been successfully pretreated in the past (Clark and Mackie 1987).

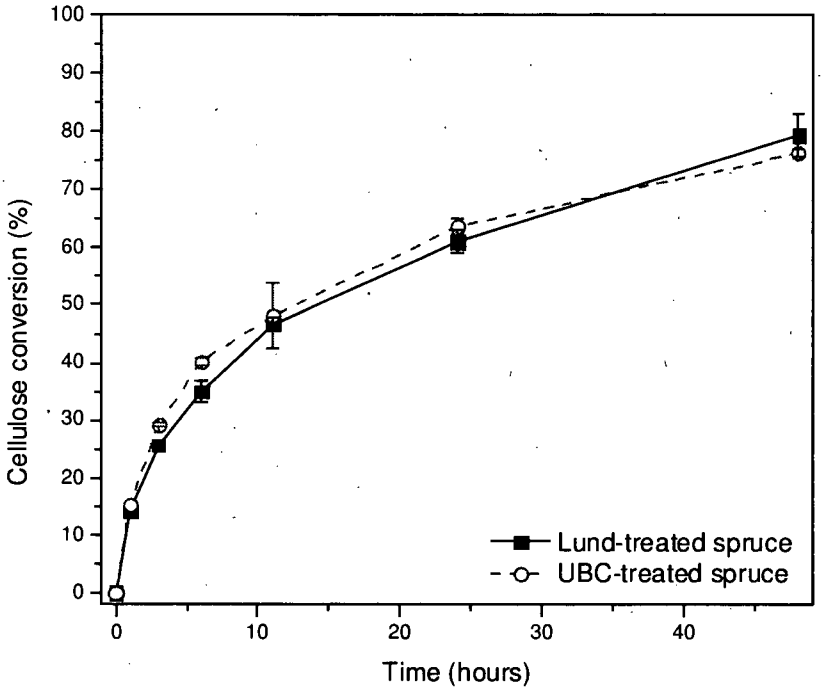

Figure 3-1. Conversion of cellulose to glucose during hydrolysis at 2% consistency of the washed solid fraction of spruce pretreated at Lund and at UBC.

3.3 Optimization of pretreatment conditions for beetle-killed lodgepole pine

After validation of the steam explosion process, it was necessary to find pretreatment conditions that gave maximal sugar recovery and ethanol yield from beetle-killed lodgepole pine. Typically, pretreatment optimization involves a complete response surface methodology study to find the optimum levels of multiple variables. This type of modelling works well on homogenous, consistent substrates. For lodgepole pine, the large amount of variability inherent in trees from an area as large and diverse as BC would require an extremely large sample size to be representative. Even with a large sample size, the effects of the beetle will change the characteristics of trees over time. For these reasons we chose to select the "optimum" pretreatment conditions that provided the best overall hexose recovery (hemicellulose and cellulose derived), cellulose hydrolysability and overall ethanol yield from a range of 7 conditions.

The seven conditions are detailed in Table 2-4, with the least severe (S1) being the optimized conditions used to pretreat Douglas-fir (Boussaid, et al. 2000) and the most severe (S7) being the conditions commonly used for Spruce (Stenberg, et al. 2000a). The other 5 conditions were compromise conditions between these two extremes. Severity is measured using the severity factor R_o, which is calculated as $R_o = te^{(T-100)/14.75}$ where t is time and T is temperature (Overend, et al. 1987).

Substrates pretreated at all seven conditions were initially evaluated for the hydrolysability and fermentability of the water-soluble stream. From these initial seven, three conditions representing low, medium and high severity were chosen. They were further characterized to determine the monomeric and oligomeric sugar profile as well as

5-hydroxymethyl furfural and furfural concentrations. The three substrates were then subjected to SSF and SHF and the overall ethanol yields compared in order to select the "optimum" pretreatment condition (Figure 3-2).

3.3.1 Composition

As is normally seen after steam pretreatment, the water-insoluble fraction of the seven substrates after pretreatment contained primarily glucose and insoluble lignin (Table 3-2). Typically, as severity increases, the amount of cellulose decreases as more cellulose is hydrolysed, and the resulting soluble glucose is separated into the water-soluble fraction. This consequently increases the ratio of lignin to glucose in the pretreated material.

Table 3-2. Composition of the washed, water-insoluble fraction of beetle-killed lodgepole pine pretreated at 7 different severities. Values in parentheses indicate deviation from the mean.

Severity		g/100g dry weight						
Code	$logR_o$	Arabinose	Galactose	Glucose	Mannose	Xylose	AIL[a]	ASL[b]
S1	3.45	0.1 (0.0)	0.1 (0.0)	58.4 (0.8)	0.5 (0.1)	0.3 (0.0)	43.9 (0.7)	0.7 (0.0)
S2 (Low)	**3.67**	**0.1** (0.0)	**0.0** (0.0)	**61.4** (0.5)	**0.7** (0.1)	**0.4** (0.1)	**44.9** (0.1)	**0.6** (0.0)
S3 (Medium)	**3.64**	**0.1** (0.0)	**0.1** (0.0)	**59.6** (0.4)	**0.8** (0.2)	**0.2** (0.1)	**46.2** (0.2)	**0.7** (0.0)
S4	3.79	0.0 (0.0)	0.0 (0.0)	53.6 (0.6)	0.2 (0.0)	0.2 (0.0)	44.4 (0.3)	0.7 (0.0)
S5	3.94	0.0 (0.0)	0.0 (0.0)	56.8 (0.0)	0.2 (0.2)	0.2 (0.2)	40.3 (0.8)	0.6 (0.1)
S6	3.94	0.0 (0.0)	0.0 (0.0)	54.2 (0.3)	0.3 (0.2)	0.1 (0.2)	44.9 (0.4)	0.8 (0.1)
S7 (High)	**4.09**	**0.0** (0.0)	**0.0** (0.0)	**46.5** (0.4)	**0.0** (0.0)	**0.0** (0.0)	**58.1** (0.3)	**1.0** (0.0)

[a]Acid insoluble (Klason) lignin
[b]Acid soluble lignin

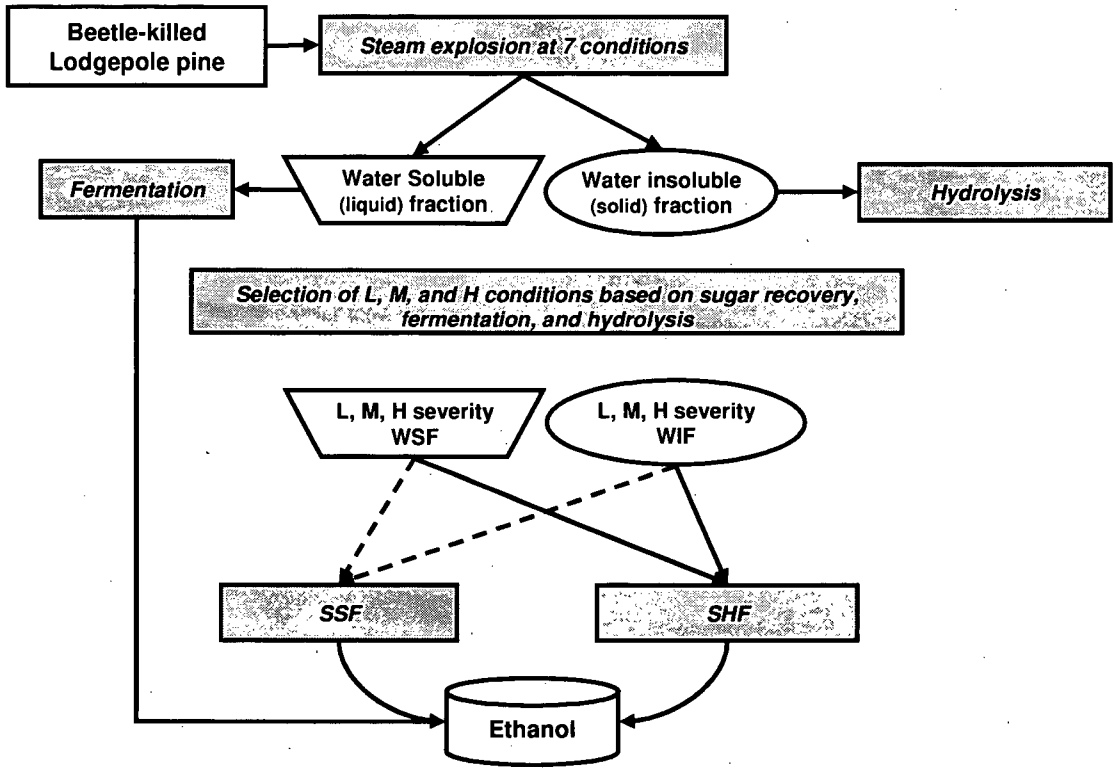

Figure 3-2. Pretreatment optimization of beetle-killed lodgepole pine.

3.3.1.1 Sugar recovery

The total recovery of sugars was derived by adding the amount of sugar found in the water-soluble and insoluble streams (after pretreatment), and the liquids recovered from washing the steam gun and from washing the water-insoluble fraction. As expected, the recovery was lower at higher severity pretreatments (Table 3-3). Figure 3-3 shows the relationship between total hexose (glucose, mannose and galactose) and pentose (arabinose and xylose) recovery and the logarithm of the severity factor R_o. There is a good correlation between the severity factor and the amount of sugar recovered (R^2 = 0.74 for pentoses, and 0.96 for hexoses) despite the fact that the acid concentration is not factored into the R_o value. The pentose recovery curve is much steeper than the hexose curve, showing the increased susceptibility of hemicellulosic sugars to degradation under severe conditions (Sjöström 1993). Although *S. cerevisiae* cannot ferment pentoses, minimizing their degradation is still important as degradation products such as furfural can inhibit the yeast during fermentation (Taherzadeh, et al. 1997).

Table 3-3. Sugar recovery after SO_2-catalyzed steam pretreatment of beetle-killed lodgepole pine at 7 different severities.

	Sugar recovery (% of original sugars)				
Severity	Arabinose	Galactose	Glucose	Mannose	Xylose
S1	83	85	100	75	70
S2 (Low)	**84**	**99**	**100**	**90**	**85**
S3 (Medium)	**78**	**94**	**100**	**81**	**73**
S4	66	79	105	70	61
S5	58	70	99	60	55
S6	53	65	100	57	48
S7 (High)	**50**	**69**	**97**	**53**	**41**

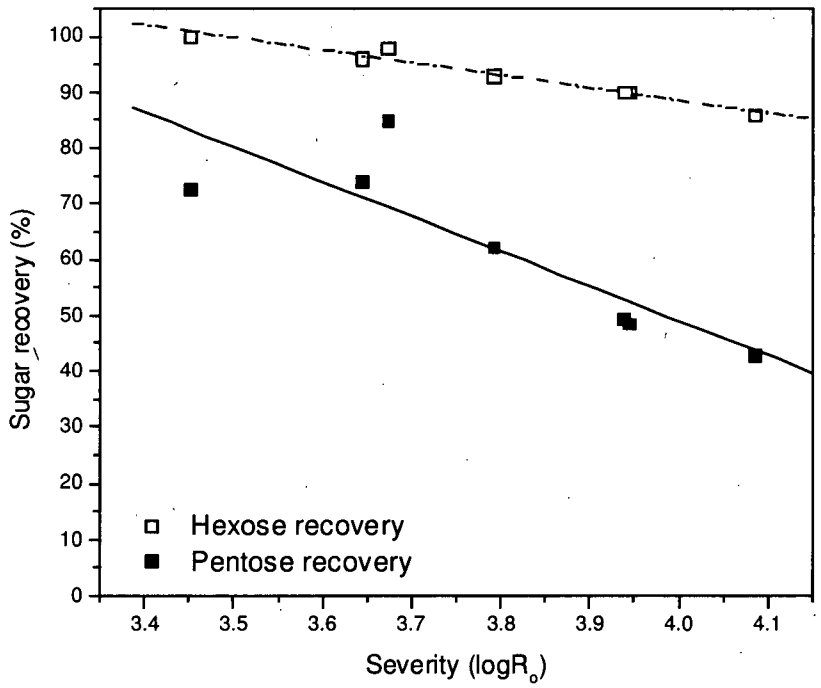

Figure 3-3. Relationship of pretreatment severity to hexose and pentose recovery after SO_2-catalyzed steam explosion of beetle-killed lodgepole pine at 7 different conditions.

3.3.2 Hydrolysis of beetle-killed pine pretreated at 7 different severities

Hydrolysis of the water-washed insoluble fraction at 2% consistency revealed that even the lowest severity pretreatments (S1 and S2) produced substrates from which 69-73% of the cellulose was hydrolysed to glucose after 72 hours (Figure 3-4). The highest conversion, 96%, was from the substrate pretreated at the highest severity (S7), clearly demonstrating the relationship between pretreatment severity and hydrolytic performance. The more severe the pretreatment, the more lignin is removed from the surface of the cellulose, increasing the accessibility for cellulases. Figure 3-5 shows the relationship between the severity factor, log R_o, and hydrolytic conversion. In this case, the R^2 value for the linear relationship is only 0.41, indicating that the severity factor may not be an accurate indicator of hydrolysability since it does not factor in the acid concentration. Ramos and Saddler observed that for pretreated Douglas-fir the SO_2 concentration was a significant indicator of the degree of hydrolysability (1994). For the beetle-killed lodgepole pine, however, the three substrates pretreatments with lower SO_2 concentrations (2%, S4, S5, and S6), were hydrolysed as well as or better than substrates with higher SO_2. This may indicate that the effect of SO_2 may be variable and specific to the feedstock used.

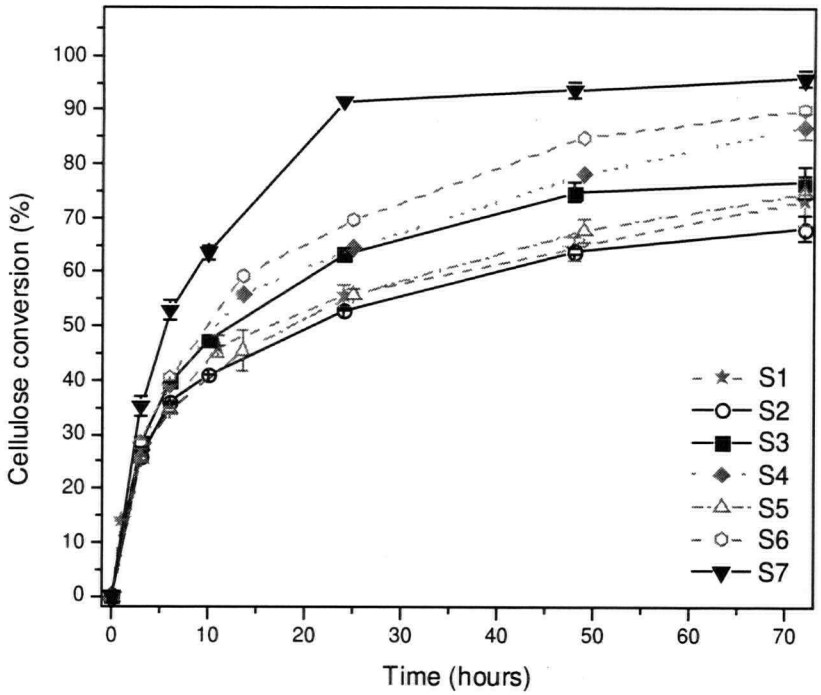

Figure 3-4. Conversion of cellulose to glucose during hydrolysis at 2% consistency of the washed solid fraction of beetle-killed (BK) lodgepole pine pretreated at 7 different conditions.

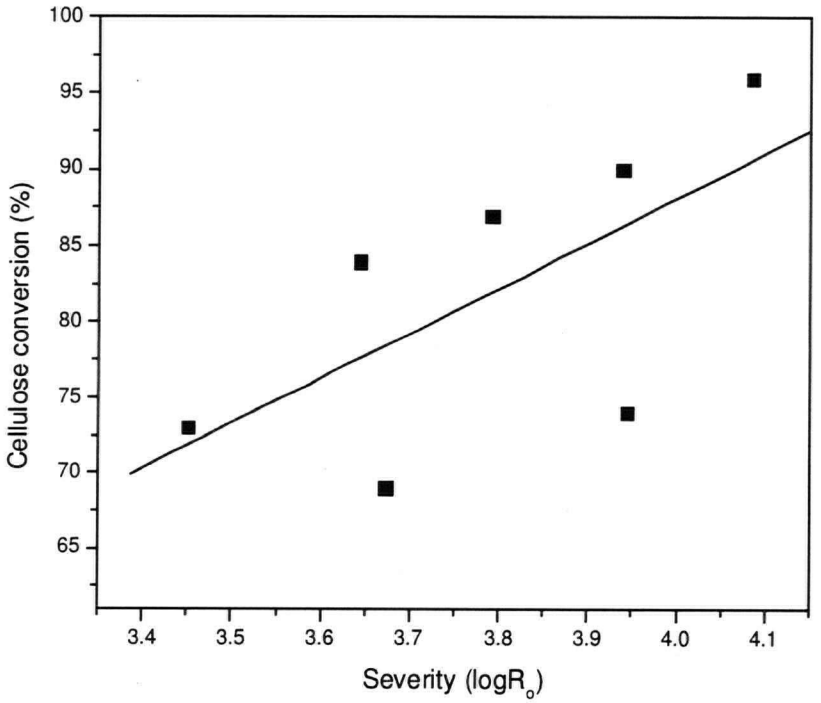

Figure 3-5. Relation of pretreatment severity to the conversion of cellulose to glucose after 72 hours of hydrolysis of the water-insoluble fraction produced after SO_2-catalyzed steam explosion of beetle-killed lodgepole pine at 7 different conditions.

3.3.3 Fermentation of beetle-killed pine pretreated at 7 different severities

Fermentation of the water-soluble streams of all seven substrates resulted in 4 of the substrates fermenting similarly well (S1, S2, S3, S5), and the other three (S4, S6, S7) fermenting poorly (Figure 3-6). For those that actually did ferment, all of the sugar was consumed after 26 hours, whereas only a third of the fermentable sugars were consumed in the same amount of time for the other three substrates. This could indicate that there was some kind of threshold concentration of inhibitory compounds above which fermentation will not proceed. It is likely that these inhibitory compounds are sugar degradation products such as furfural or 5-hydroxymethyl furfural (HMF), which must be completely metabolized by yeast before any growth can occur (Taherzadeh, et al. 2000). It should also be noted that for substrates pretreated at S1, S2, S3, and S5 conditions, the conversion of fermentable sugars to ethanol was between 29 and 37%, corresponding to 57 to 73% theoretical ethanol yield. These yields are slightly lower than expected, and might be due to inhibitory compounds.

Stenberg et al. (1998) found that for fermentation of SO_2-catalysed steam pretreated mixed spruce and pine, pretreatment temperature and not the overall severity factor affected substrate fermentability. This seemed to be the same case for our samples, with S1, S2, S3 and S5 samples pretreated at 200°C or lower, and S4, S6 and S7 pretreated at 205°C or higher. However, Stenberg et al. found that fermentation of the water-soluble stream from substrates pretreated at high severities (5.0) and temperature (230°C) still provided 78% of theoretical ethanol yield after 20 hours, albeit with the addition of nutrients and supplemental glucose.

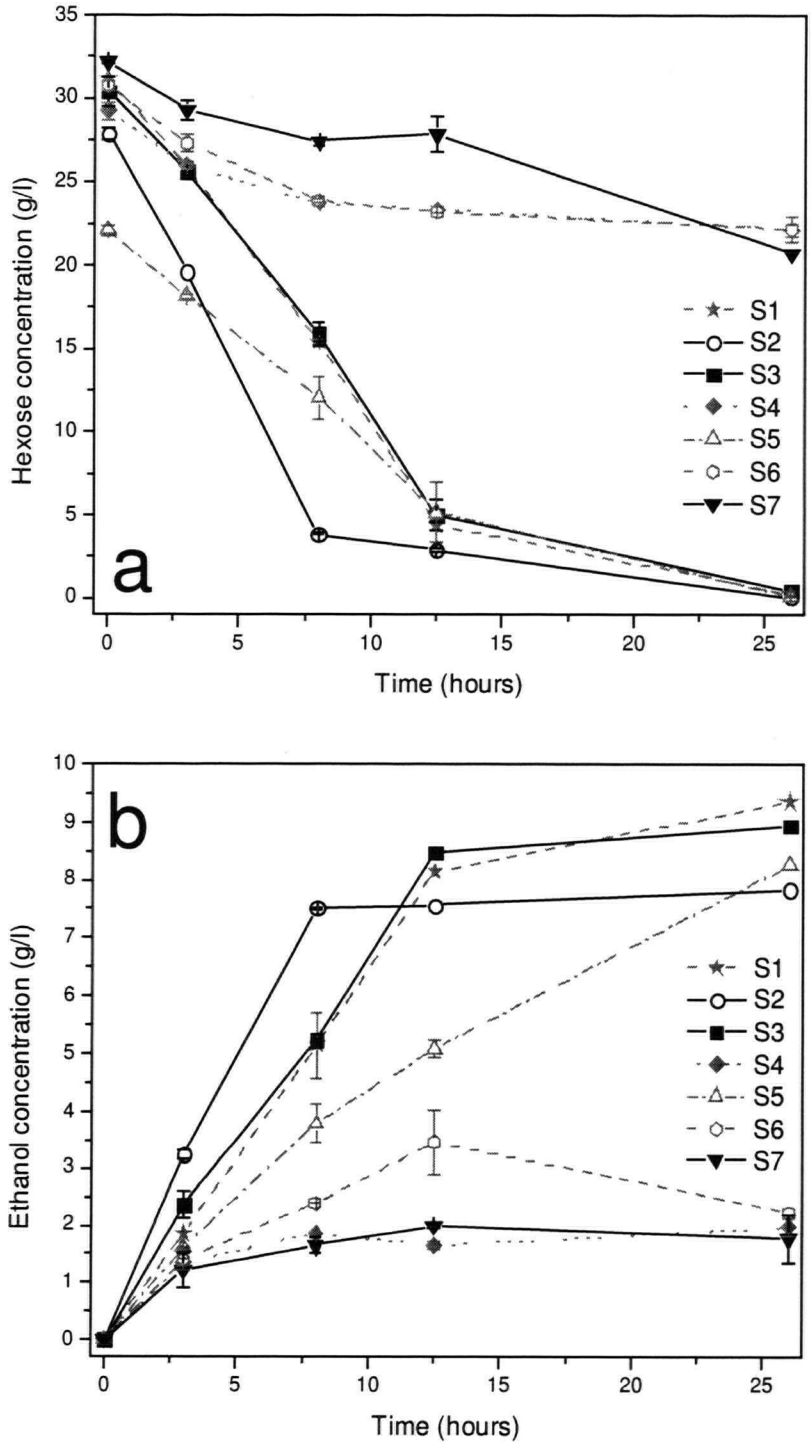

Figure 3-6. Hexose consumption (a) and ethanol production (b) during fermentation of the water-soluble fraction of beetle-killed (BK) lodgepole pine pretreated at 7 different conditions.

3.3.4 Selection of three pretreatment conditions

The previous section showed the characteristics of substrates produced at seven different pretreatment severities. This work also showed the optimum conditions for maximum hydrolysability and sugar recovery. However, the most important parameter is to determine the overall ethanol yield obtained after either SHF or SSF. To find the pretreatment conditions that provided the highest ethanol yields, three conditions were selected (described below), and are hereafter referred to as low, medium and high severity (Table 3-4). S2 was selected to represent low (L) severity, as it provided the highest hexose recovery (98%) and the resulting substrate was fermentable. S7 was chosen as the high (H) severity condition. This substrate had the lowest hexose recovery (86%) and was not well fermented, but provided 96% hydrolytic conversion. Finally, S3 was chosen to be the medium (M) severity pretreatment as the resulting substrate provided excellent hexose recovery after pretreatment (96%), 84% conversion after hydrolysis, and was readily fermented.

Table 3-4. Summary of sugar recovery, hydrolysis, and fermentation results obtained from beetle-killed lodgepole pine pretreated at 7 severities.

Severity	Pretreatment severity ($logR_o$)	Hexose recovery (%)	Hydrolytic conversion[a] (%)	Fermentation conversion[b] (%)
S1	3.45	100	73	30
S2 (Low)	**3.67**	**98**	**69**	**28**
S3 (Medium)	**3.64**	**96**	**84**	**37**
S4	3.79	93	87	7
S5	3.94	90	74	29
S6	3.94	90	90	10
S7 (High)	**4.09**	**86**	**96**	**6**

[a]Percentage of pretreated cellulose converted to glucose after 72 hours of hydrolysis
[b]Percentage of hexoses converted to ethanol

3.3.4.1 Monomeric and oligomeric sugars

It is well established that as pretreatment severity increases, there is increased glycolysis of polymeric, hemicellulosic sugars to monomers (Ramos 2003). This is partially evident after pretreatment of beetle-killed pine at low, medium and high severity (Figure 3-8), though only the pentose sugars xylose and arabinose show a significant drop in oligomeric sugar concentration as severity increases. However, it is apparent that pentose sugars are the most affected by pretreatment at all three severities, with the lowest proportion of oligomers remaining after pretreatment at all three severities. The increased susceptibility of arabinoglucuronoxylans to acid hydrolysis is responsible for the drop in the pentosan content. However, since arabinoglucuronoxylan constitutes only 5-10% of softwood, compared to galactoglucomannan, which represents approximately 20% (Sjöström 1993), this sugar loss does not contribute appreciably to the total sugar recovery.

With increased pretreatment severity we observed increased degradation and subsequent loss of monomeric sugars. The amount of sugars present in the water-soluble fraction relative to the sugars present in the original wood shows the proportion of sugars that have been degraded (Figure 3-8). For the hemicellulosic sugars (arabinose, galactose, xylose and mannose), the amount of sugar decreases as pretreatment severity increases. The concentration of glucose in the water-soluble stream increases as the cellulose in the insoluble fraction is hydrolysed to glucose. Correspondingly, there is a decrease in glucose recovered from the water-insoluble fraction as severity increases (Figure 3-9). Likewise, mannose and galactose are present in the insoluble fraction pretreated at low and high severities, but have been completely solubilized at high severity. This is typical

of softwood pretreatment, as a similar decline in insoluble galactose and mannose content was seen after pretreatment of Douglas-fir at low, medium and high severity (Boussaid, et al. 2000).

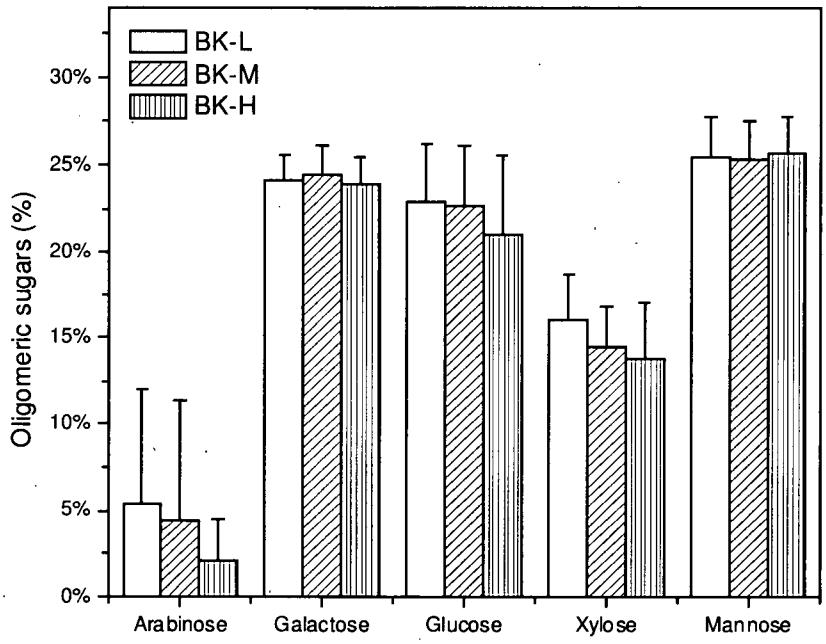

Figure 3-7. Amount of oligomeric sugars as a percentage of the total sugars of each type present in the water-soluble fraction after pretreatment of beetle-killed (BK) lodgepole pine at low (L), medium (M) and high (H) severities.

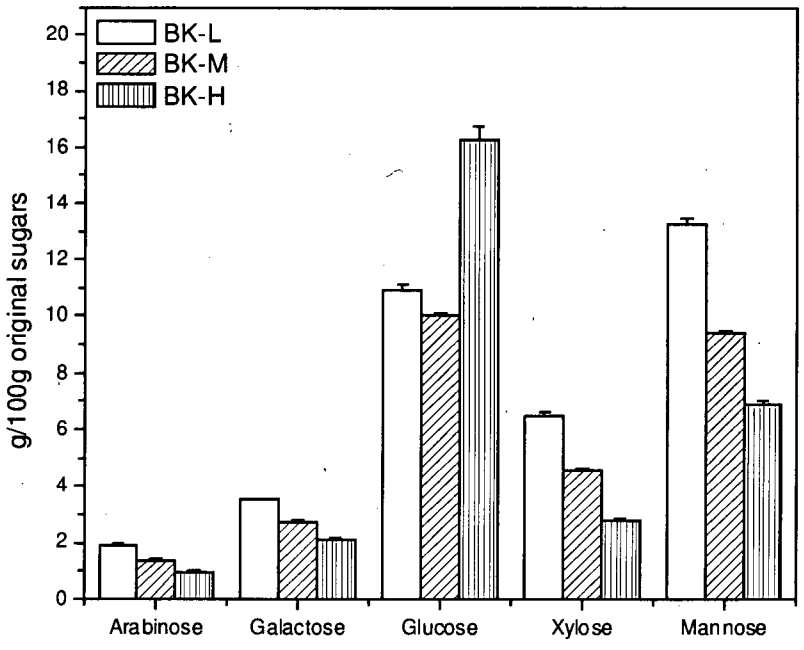

Figure 3-8. Sugar recovery from the water-soluble fraction after pretreatment of beetle-killed (BK) lodgepole pine at low (L), medium (M) and high (H) severities.

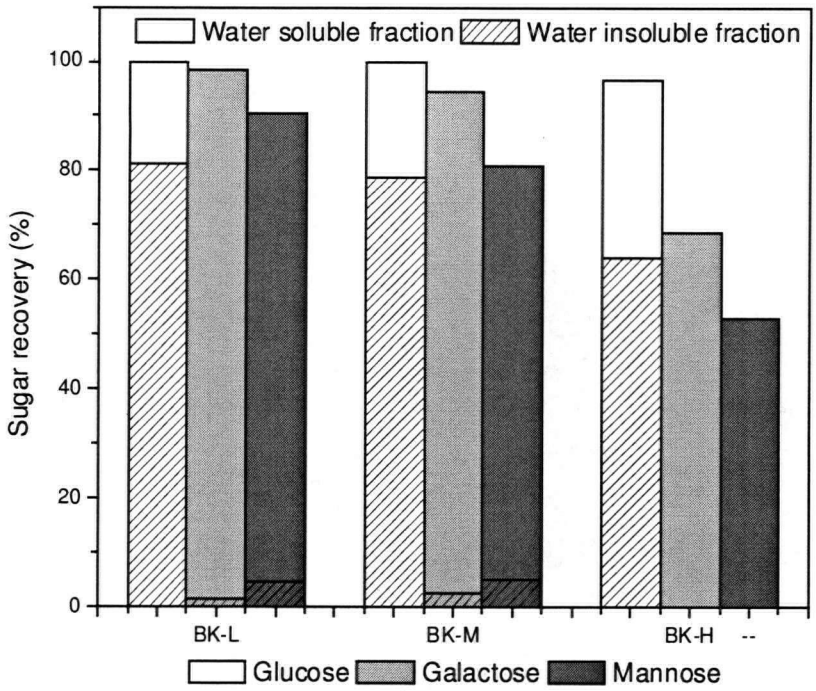

Figure 3-9. Glucose, galactose and mannose recovered in water-insoluble and soluble fractions after pretreatment of beetle-killed (BK) lodgepole pine at low (L), medium (M) and high (H) severities.

3.3.4.2 HMF and furfural analysis

Furfural and 5-hydroxymethyl furfural (HMF) are produced during pretreatment at high temperatures by dehydration reactions of pentose and hexose sugars, respectively (Taherzadeh, et al. 2000). At sufficiently high levels, they are known to be inhibitory to *S. cerevisiae* (Taherzadeh, et al. 1997). Furfural and HMF concentrations were measured in the water-soluble fraction after pretreatment of beetle-killed pine at low, medium and high severity conditions (Table 3-5). The concentration of HMF was more than twice as high for pine treated at high severity compared to the substrate pretreated at low severity. However, the furfural concentrations were similar at all three severity levels. These concentrations are typical of softwoods, as Stenberg et al. (2000a) observed a furfural concentration of 1.3 g/L and a similar HMF concentration of 2.0 g/l after pretreatment of spruce at 215°C, 5 min, 2.8% m.c. SO_2 ($logR_o = 4.1$),

Table 3-5. Concentrations of 5-hydroxymethyl furfural (HMF) and furfural in the water-soluble fraction (WSF) after pretreatment of beetle-killed lodgepole pine at low, medium, and high severities. Deviation from the mean is shown in parentheses.

	Concentration in WSF after pretreatment (g/l)	
Severity	HMF	Furfural
Low	1.36 (0.05)	0.81 (0.04)
Medium	2.23 (0.16)	1.04 (0.05)
High	3.39 (0.17)	0.99 (0.05)

It is recognized that longer pretreatment residence times lead to dilution of the water-soluble fraction with steam, so it is useful to express HMF and furfural as a percentage of the total sugars in the raw material (Figure 3-10). When the results are expressed this way, it is apparent that the low and medium severity pretreatments generated similar amounts of HMF relative to the starting sugars, while the high severity pretreatments

produced significantly more. This high concentration of HMF was probably responsible for the poor fermentability of the water-soluble fraction. Furfural concentrations did not differ between pretreatment severities, indicating that the pentose sugars from which furfural is derived were affected equally by all three severities. However, the HMF-forming hexose sugars seemed to be much more susceptible to degradation at high severity conditions. Pentoses are known to be degraded at lower severities than hexoses, since pentose-containing hemicellulose is solubilized more quickly as severity increases (Bura, et al. 2002). Once the sugars are solubilized, they are much more susceptible to degradation. Thus, even at low severity, the maximum amount of furfural has already formed, while increased degradation of hexoses to HMF is still occurring under high severity conditions.

The amount of HMF and furfural formed did not seem to correspond with the increasing loss of hexose and pentose sugars as severity increased (Figure 3-8), especially furfural. Although the amount of pentose sugars decreased with severity, the amount of furfural formed did not increase accordingly, but rather remained constant. HMF similarly did not increase from low to medium severity, even though there was a decrease in the amount of galactose and mannose. This indicates only a certain amount of sugars were degraded to HMF and furfural, with the remainder subject to oxidation and other decomposition process.

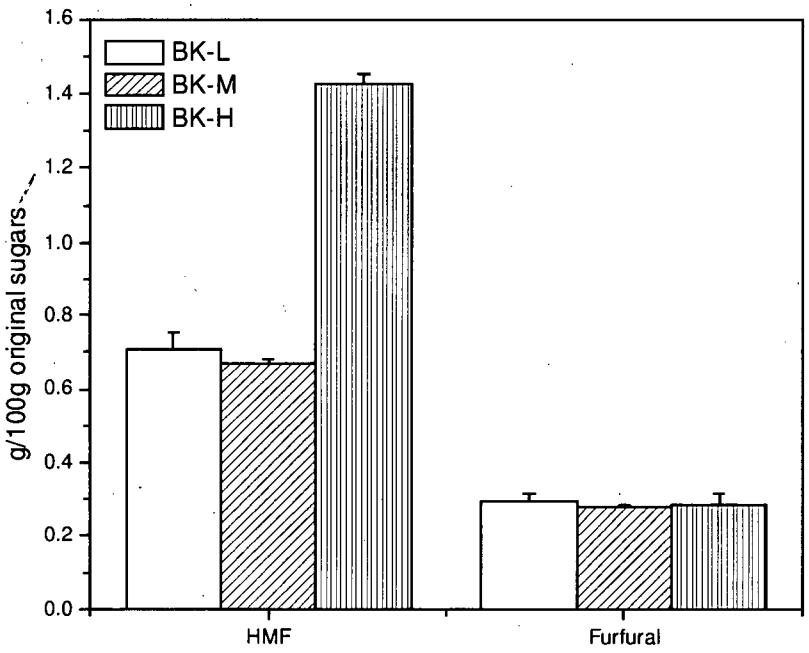

Figure 3-10. 5-hydroxymethyl furfural (HMF) and furfural concentrations in the water-soluble fraction after pretreatment of beetle-killed (BK) lodgepole pine at low (L), medium (M) and high (H) severities.

3.3.5 Separate hydrolysis and fermentation of beetle-killed pine

Separate hydrolysis and fermentation (SHF) combines the water-soluble and insoluble fractions at 5% consistency, and has the advantage of allowing hydrolysis and fermentation to be carried out at their optimum temperature and pH. After 72 hours of hydrolysis, cellulose to glucose conversions of 69, 75 and 77% were observed for low, medium, and high-severity pretreated substrates (Figure 3-11). Each of these conversions is lower than their respective conversions after 2% consistency hydrolysis. This is likely due to increased end product inhibition (Holtzapple, et al. 1990) and inhibition from degradation products generated during pretreatment which are present in the water-soluble fraction (Robinson 2003; Tengborg, et al. 2001b).

After fermentation, higher ethanol concentrations were achieved from the medium and high-severity pretreated material than from the substrate pretreated at low severity. Experimental yields for low, medium, and high-severity pretreated material were 73, 75, and 85% respectively of the theoretical maximum yield of ethanol from the pretreated material. The incomplete initial hydrolysis and varied sugar recoveries after pretreatment result in similar overall yields of 72, 72, and 73% of theoretical ethanol based on the amount of hexoses in the raw material for low, medium, and high-severity pretreated substrates, the equivalent of 228, 229, 231g ethanol per kg of raw material. The yields are remarkably similar, and show that even with variation in hydrolysability and sugar recovery, the variables balance each other out.

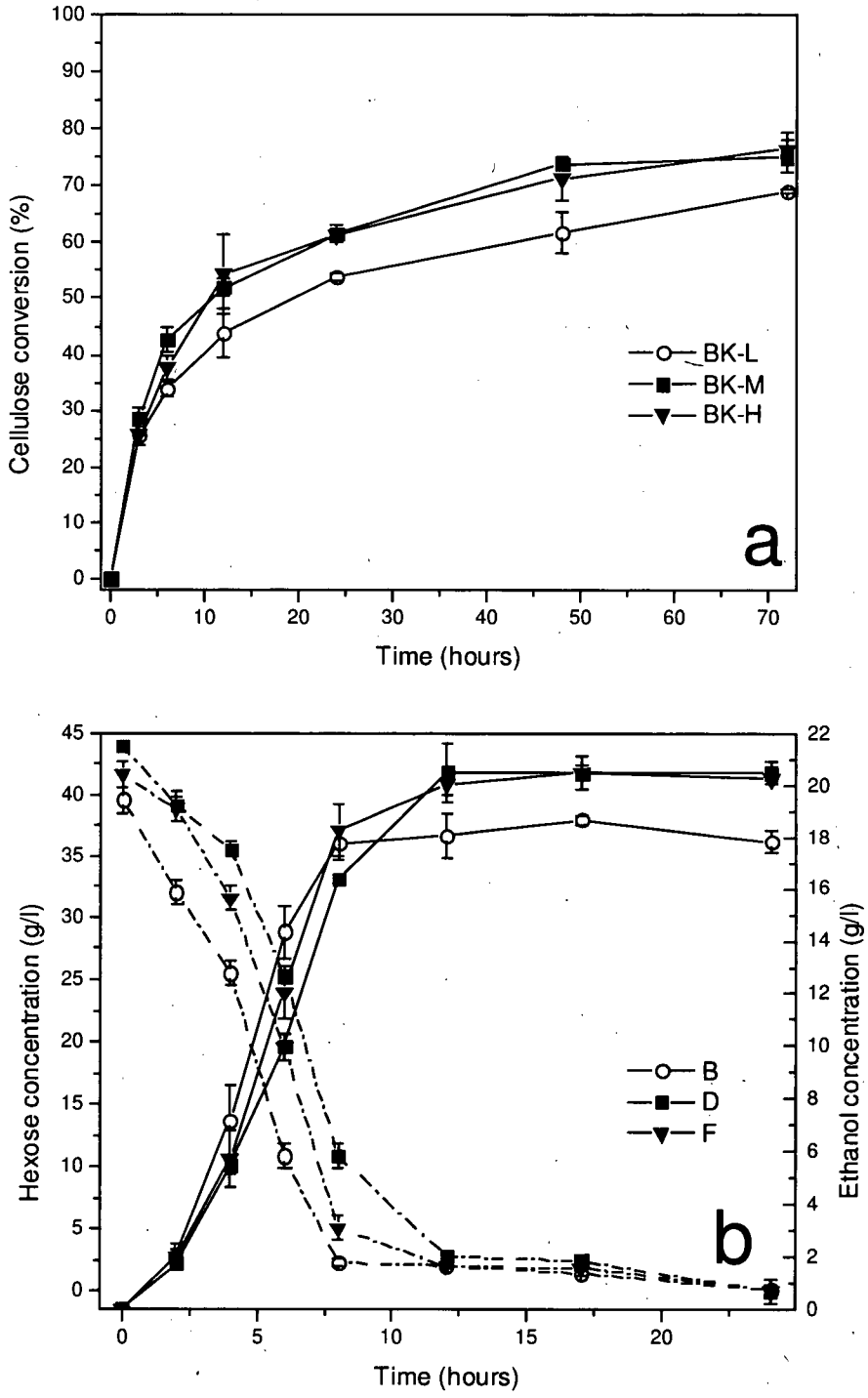

Figure 3-11. Hydrolysis (a) and fermentation showing hexose consumption (dotted lines) and ethanol production (solid lines) (b) during SHF of beetle-killed (BK) lodgepole pine pretreated at low (L), medium (M) and high (H) severities.

3.3.6 Development of simultaneous saccharification and fermentation of beetle-killed pine

Although SHF of pretreated beetle-killed pine resulted in high overall ethanol yields, SSF is often known to provide even better yields due to removal of the effect of end-product inhibition on hydrolysis (Söderstrom, et al. 2005). However, SSF using the method previously used at UBC for agricultural residues (Bura 2004) resulted in very low ethanol yields from the pretreated beetle-killed pine. As will be described in detail below, modification of the method to include a prehydrolysis step and addition of supplemental nutrients resulted in dramatically higher ethanol yields.

3.3.6.1 Simultaneous saccharification and fermentation method development

Simultaneous saccharification and fermentation (SSF) often allows the time to produce a given amount of ethanol to be reduced by removing the effects of carbohydrate end-product inhibition. Glucose and cellobiose produced during hydrolysis can inhibit cellulase enzymes, but during SSF the glucose is immediately consumed by yeast, removing its inhibitory effect (Holtzapple, et al. 1990). On the other hand, hydrolytic enzymes can be inhibited by high concentrations of ethanol (Wu and Lee 1997), although this inhibition is weaker than that of glucose or cellobiose (Holtzapple, et al. 1990).

For method development of SSF, only the material pretreated at medium severity was used. The aim of the process was to evaluate whether adequate ethanol yields from SSF were possible on this substrate. Established procedures such as nutrient supplementation, pH monitoring and maintenance, and prehydrolysis were employed.

3.3.6.1.1 Effect of pH and yeast concentration on hexose consumption

Without modification to the process, there was very little ethanol produced during SSF of any of the three substrates. It was postulated that perhaps the pH of the solution was decreasing over the course of the experiment, and hindering either hydrolysis or fermentation. Another possibility was that there was a lower concentration of productive yeast for some reason, and if fresh yeast were added the sugar consumption might increase. The consumption of hexoses was monitored over 48 hours of SSF (Figure 3-12). For the first sample, the pH was maintained at pH 5 by measuring the pH at every time point and adjusting it if necessary. The pH dropped to 4.5 within the first 4 hours, but remained constant after that. The drop in pH did not appear to be a problem, however, as the control sample without pH maintenance showed higher sugar consumption after 24 hours. The third sample was given a second "dose" of yeast after 10 hours. This appeared to have a slightly positive effect, although very few of the hexoses had been consumed after 48 hours.

3.3.6.1.2 Effect of nutrient addition and prehydrolysis on ethanol yield

Since it appeared that neither the pH or yeast concentration was entirely responsible for the low ethanol yields, the effect of nutrient addition along with the inclusion of a prehydrolysis step was investigated. Both nutrient addition and prehydrolysis have been shown to improve the ethanol yield during SSF (Stenberg, et al. 2000b; Varga, et al. 2004). Prehydrolysis entails raising the temperature for a period of time to maximize hydrolytic conversion before adding yeast and reducing the temperature (Rudolf, et al. 2005). The nutrients chosen were based on those added for SSF of steam pretreated spruce: $NH_4H_2PO_4$, $MgSO_4 \cdot 7H_2O$, yeast extract and peptone (Rudolf, et al. 2005;

Stenberg, et al. 2000b). Stenberg et al. (2000b) found that during the SSF of steam-pretreated spruce, the addition of yeast extract resulted in a cell concentration five times higher than that obtained without supplementation. This high yeast concentration resulted in immediate production of ethanol, whereas without addition of yeast extract the ethanol concentration remained below 5 g/l for 55 hours before finally increasing to a similar final ethanol concentration.

Prehydrolysis resulted in much higher ethanol concentrations after 24 hours of SSF than when the yeast and enzyme were added at the same time (Figure 3-13). However, there did not appear to be a difference between 12 or 6 hours of prehydrolysis. The addition of nutrients slightly increased the ethanol yield for both the 12 and 6 hour pre-hydrolysed samples but did not have an effect on the control sample that did not undergo prehydrolysis (Figure 3-13).

3.3.6.1.3 Effect of second addition of yeast on ethanol yield

While the addition of a prehydrolysis step and nutrients improved the ethanol yield substantially, it was thought that a second addition of yeast might further improve yields. Samples prehydrolysed for 0, 6, 12 or 24 hours were given an extra dose of yeast after 11 hours of SSF (Figure 3-14). The extra yeast did not appear to have any effect on the prehydrolysed samples, but dramatically increased the ethanol yield for the control sample. A likely explanation for this is that yeast require a certain level of fermentable sugars before fermentation can begin. Prehydrolysis achieves this by providing a high concentration of glucose in the solution before adding the yeast, which can then immediately begin fermentation. Without prehydrolysis, the second addition of yeast was more successful than the first since there was an increased concentration of glucose present from the ongoing hydrolysis. However, the final ethanol yield was still lower than that achieved after 6 hours of prehydrolysis with no extra addition of yeast.

3.3.6.1.4 Conclusion

Based on these results, a 6-hour prehydrolysis and nutrient supplementation was included in the standard SSF procedure for all of the beetle-killed and healthy pine samples studied. This was thought to be the best way to increase ethanol yield with minimal additional steps or modification to the original procedure.

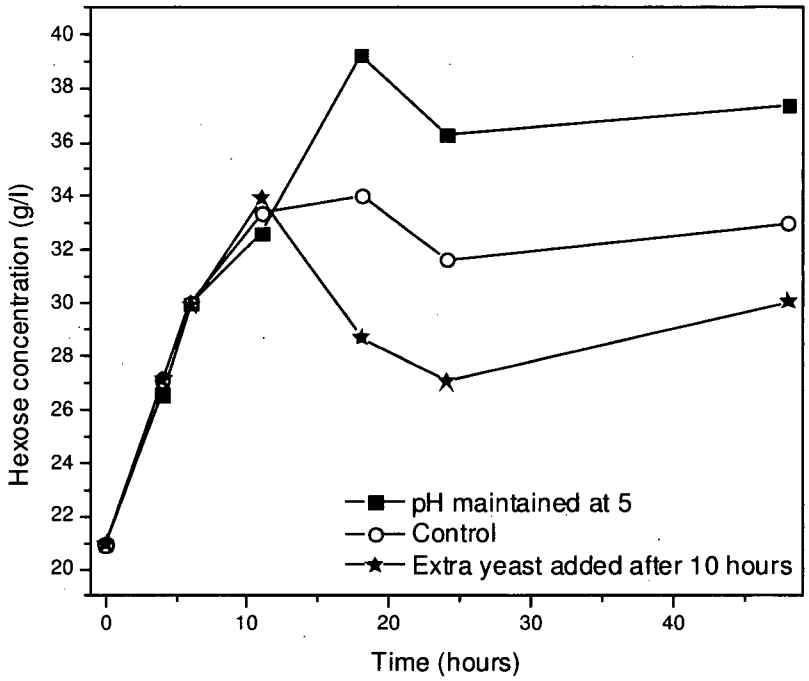

Figure 3-12. Hexose concentration during SSF of beetle-killed pine pretreated at medium (M) severity during simultaneous saccharification and fermentation (SSF) with no prehydrolysis. 5 g/l yeast was added at time 0 for all three experiments.

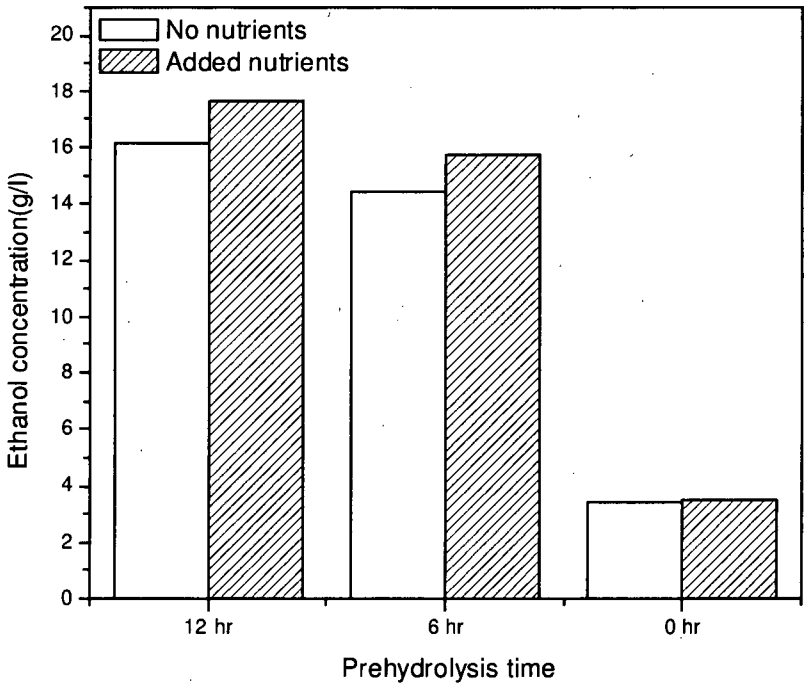

Figure 3-13. Effect of nutrient addition and prehydrolysis on ethanol concentration after 24 hours of simultaneous saccharification and fermentation (SSF) of beetle-killed pine pretreated at medium (M) severity.

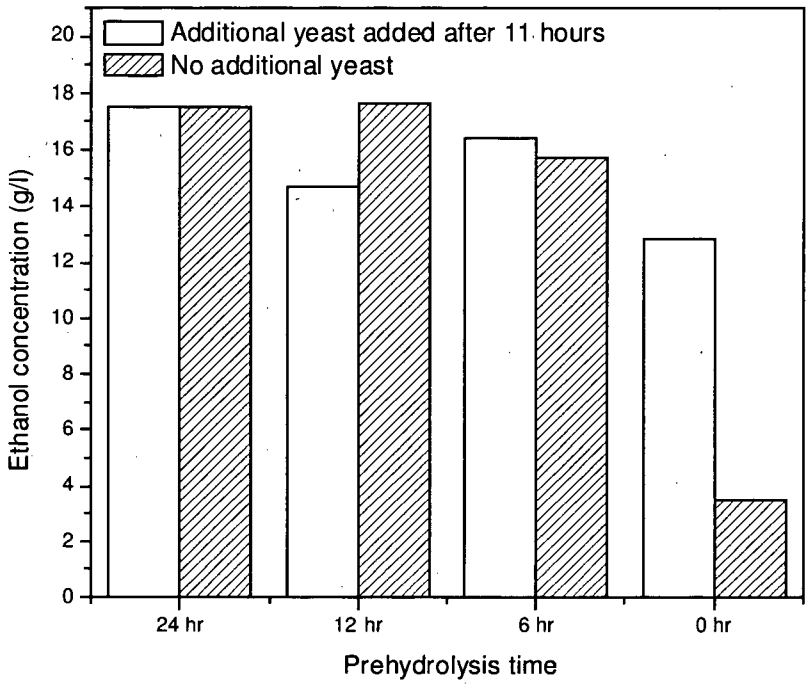

Figure 3-14. Effect of additional yeast addition and prehydrolysis on ethanol concentration after 24 hours of simultaneous saccharification and fermentation (SSF) of beetle-killed pine pretreated at medium (M) severity. All samples were supplemented with nutrients.

3.3.6.2 Simultaneous saccharification and fermentation of beetle-killed pine

3.3.6.2.1 Ethanol production

After modification of the SSF procedure, the ethanol yields were comparable to or better than those achieved after SHF (Figure 3-15). Experimental ethanol yields after SSF were 62%, 80%, and 75% of the theoretical maximum for wood pretreated at low, medium, and high severity (based on starting hexose concentration in pretreated material). After consideration of the sugar recovery after pretreatment, the overall ethanol yield was 61, 77, and 65% of the theoretical maximum yield from the raw material for low, medium, and high severity-pretreated material, the equivalent of 193, 244, and 205 g ethanol per kg raw material.

3.3.6.2.2 HMF and furfural consumption

After 6 hours of SSF following the prehydrolysis, all of the furfural had been consumed, with a slightly faster rate of consumption in the low-severity pretreated substrate (Figure 3-16). More time was required for HMF to be completely consumed, but after 24 hours none remained (Figure 3-17). Again, HMF in the low-severity pretreated material was consumed at the fastest rate. Interestingly, even though the starting concentration of HMF in the high-severity pretreated material was approximately twice that of the medium-severity pretreated substrate, it did not take any longer to be consumed.

These results confirm that yeast first consume furfural, and then HMF (Taherzadeh, et al. 2000). However, HMF is a stronger inhibitor of *S. cerevisiae* than furfural, which requires twice the concentration to attain the same reduction in productivity (Larsson, et al. 1999). It has been shown that cell growth is inhibited until all of the HMF and furfural

is consumed (Taherzadeh, et al. 2000), although once consumed, neither furfural nor HMF affect the final yield of ethanol. However, it did affect the rate at which ethanol was produced (Larsson, et al. 1999).

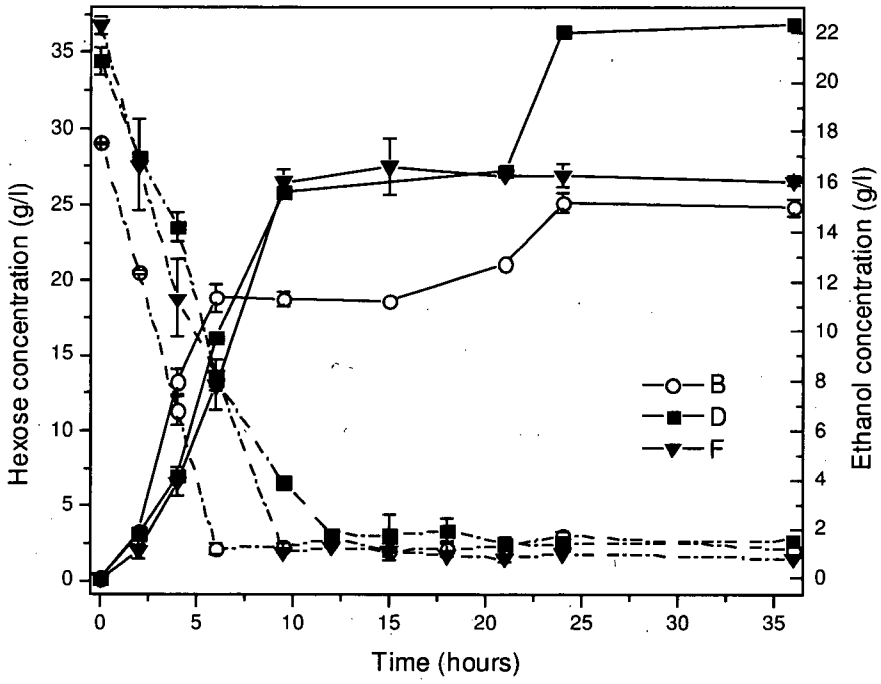

Figure 3-15. Hexose consumption (dotted lines) and ethanol production (solid lines) during SSF of beetle-killed (BK) lodgepole pine pretreated at low (L), medium (M) and high (H) severities. 6 hour prehydrolysis not shown.

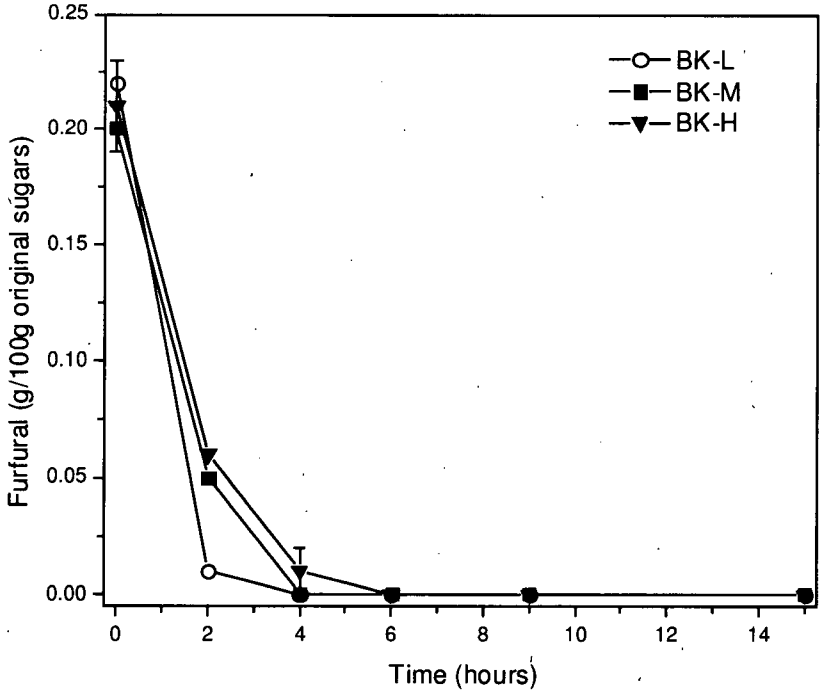

Figure 3-16. Consumption of furfural during simultaneous saccharification and fermentation (SSF) of beetle-killed (BK) lodgepole pine pretreated at low (L), medium (M) and high (H) severities. 6 hour prehydrolysis not shown.

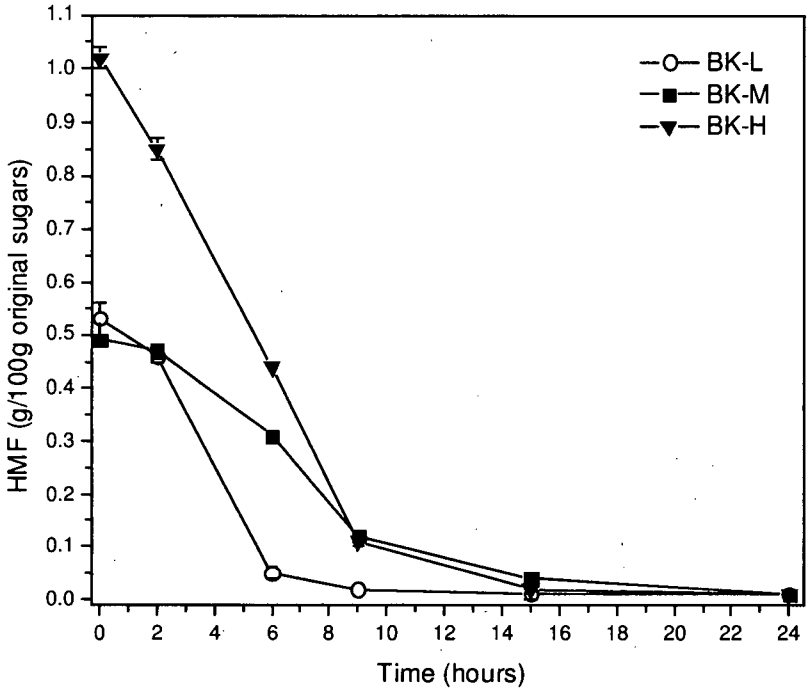

Figure 3-17. Consumption of 5-hydroxymethyl furfural (HMF) during simultaneous saccharification and fermentation (SSF) of beetle-killed (BK) lodgepole pine pretreated at low (L), medium (M) and high (H) severities. 6 hour prehydrolysis not shown.

3.3.7 Comparison of separate and simultaneous hydrolysis and fermentation

With a total time of only 30 hours for SSF compared to 96 hours for SHF, SSF required lower energy input for similar yields, particularly at the medium severity-pretreated material. However, the ethanol yield was only higher after SSF for the pine pretreated at medium severity and not at low or high severity conditions. The low-severity pretreated material likely required the longer hydrolysis time during SHF to convert an increased amount of cellulose to glucose. The material pretreated at high severity had reduced sugar recovery after pretreatment so its potential yield is lower. Medium-severity pretreated wood had the ideal balance of hydrolysability and sugar recovery and therefore provided the highest overall ethanol yield. SHF yields are nearly the same for all three substrates, likely because the extended hydrolysis time allows maximum hydrolytic conversion.

Stenberg et al. (2000a) compared SSF to SHF, and found that under similar conditions to those used in the work presented here, SHF of steam-pretreated spruce provided 40% of the theoretical maximum overall ethanol yield, while SSF yielded 60% of the theoretical value. In addition, the productivity of the process was increased, since the time required was reduced by half (Stenberg, et al. 2000a). In terms of ethanol yield from raw material, the observed ethanol yield of 244 g ethanol/kg d.w. after SSF of beetle-killed lodgepole pine is comparable to the 210 g ethanol/kg d.w. observed by Stenberg et al. (2000b) after SSF of steam pretreated spruce in a 1-L fermentor at the same consistency and enzyme loading. This indicates that beetle-killed lodgepole pine as a bioconversion feedstock may be as good as or better than spruce.

3.3.8 Selection of the optimum pretreatment conditions

For the highest overall ethanol yield from beetle-killed lodgepole pine, the optimum pretreatment (of the three that were investigated) is clearly the medium severity conditions (200°C, 5 min, 4% SO_2). Pretreatment at these conditions provided 229 g ethanol/kg raw material after SHF, and 244 g/kg after SSF. These yields correspond to 72 and 77% of the theoretical maximum ethanol yield from the original material. Compared to pretreatment optima for other substrates, 200°C, 5 min, 4% SO_2 is somewhat mild. For spruce, 215°C, 5 min, 3% w/w moisture SO_2 is optimum (Stenberg, et al. 2000a), 210°C, 5.5 min, 4.5% SO_2 is ideal for mixed softwoods (Stenberg, et al. 1998), and 215°C, 3 min, 2.6% SO_2 for Radiata pine (Clark and Mackie 1987). The optimum for Douglas-fir is lower, at 195°C, 4.5 min, 4.5% w/w SO_2, but the substrate generated requires further delignification to provide satisfactory hydrolytic conversion (Boussaid, et al. 2000). The somewhat mild conditions chosen for beetle killed-lodgepole pine minimize the production of sugar degradation products while still allowing efficient hydrolytic conversion.

3.3.9 Conclusion

Optimization of SO_2-catalyzed steam explosion of beetle-killed lodgepole pine began with pretreatment at seven different conditions of increasing severity. As the pretreatment severity increased, sugar recovery decreased and hydrolytic conversion of the water-insoluble fraction increased. After fermentation of the water-soluble fraction, at least half of the theoretical ethanol yield was obtained from 4 of the substrates, whereas the remaining three substrates fermented poorly. Based on these results, low, medium and high-severity pretreatments were chosen representing high sugar recovery and

fermentability (low severity), high hydrolytic conversion (high severity) or both (medium severity). The concentration of HMF in the water-soluble fraction was found to increase with increasing severity, while furfural concentrations were similar for all three substrates. The increased HMF may have been the reason for the poor fermentability of the high-severity pretreated material. Despite this, however, overall ethanol yields after SHF were similar for all three substrates (72-73% of theoretical).

In order to reduce the long residence time required for SHF, an SSF procedure was developed which enabled similar ethanol concentrations to be produced in a third of the time required for SHF. Overall ethanol yields after SSF were 61, 77, and 65% of theoretical for the low, medium and high severity-pretreated substrates. Based on these results, the medium-severity pretreatment (200°C, 5 min, 4% SO_2) was chosen as the "optimum" pretreatment for beetle-killed lodgepole pine. The material pretreated at this severity was ideal for SSF, as it had high fermentable sugar recovery after pretreatment, was rapidly enzymatically hydrolysed, and contained sufficiently low levels of inhibitors so as to enable high ethanol yields during fermentation.

3.4 Robustness of pretreatment

In the future, the lodgepole pine available in British Columbia will vary considerably in different regions. As the mountain pine beetle outbreak subsides, the amount of healthy lodgepole pine available will increase over time. However, beetle outbreaks are predicted to continue to occur more frequently in the future due to global warming, so the wood supply is likely to be variable and unpredictable. A bioconversion process utilizing lodgepole pine therefore needs to be robust enough to produce ethanol from this variable supply. To assess whether the medium severity pretreatment conditions chosen in section

(50/50 healthy/beetle-killed) lodgepole pine (Figure 3-18) and compared with our previous results when these conditions were applied to beetle-killed pine.

3.4.1 Composition

Prior to pretreatment, the chemical composition of the raw pine differed slightly between beetle-killed and healthy (Table 2-3). The beetle-killed wood contained 7% less glucose and 9% more lignin than the healthy wood. However, the beetle-killed wood took up approximately 20% less SO_2 during the impregnation step of pretreatment, effectively reducing the severity of the pretreatment. This milder severity pretreatment is apparent in the composition of the washed, water-insoluble fractions, with the pretreated healthy pine having 8% less glucose and 7% more lignin than was observed in the beetle-killed wood (Table 3-6). This shows that the beetle-killed substrate lost less glucose to the water-soluble fraction, increasing the glucose content in the solids and effectively decreasing the concentration of lignin. As expected, the glucose and lignin contents in the mixed sample were between those observed in the beetle-killed and healthy substrates.

Table 3-6. Composition of the washed, water-insoluble fraction of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity. Values in parentheses represent deviation from the mean.

	g/100g dry weight						
	Arabinose	Galactose	Glucose	Mannose	Xylose	AIL[a]	ASL[b]
BK-M	0.1 (0.0)	0.1 (0.0)	59.6 (0.4)	0.8 (0.2)	0.2 (0.1)	46.2 (0.2)	0.7 (0.0)
Healthy-M	0.1 (0.0)	0.0 (0.0)	54.8 (0.4)	1.0 (0.0)	0.4 (0.0)	49.6 (0.4)	0.7 (0.0)
Mix-M	0.0 (0.0)	0.0 (0.0)	58.0 (0.6)	0.5 (0.1)	0.2 (0.1)	46.6 (0.2)	0.7 (0.0)

[a]**Acid insoluble (Klason) lignin**
[b]**Acid soluble lignin**

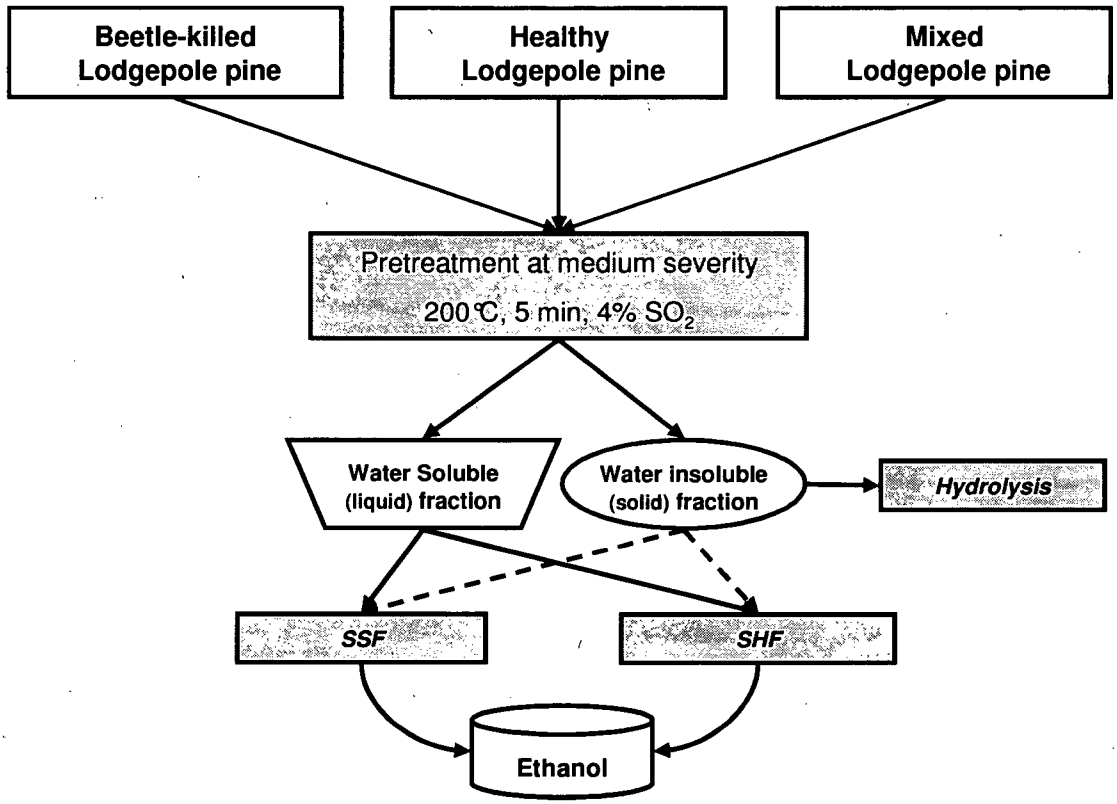

Figure 3-18. Bioconversion of beetle-killed, healthy, and mixed (50/50 beetle-killed/healthy) lodgepole pine.

3.4.1.1 Sugar recovery

After pretreatment, total hexose recovery was close to 100% for all three feedstocks, but sugar recoveries were consistently higher for beetle-killed wood (Table 3-7). This is likely a result of the reduced uptake of SO_2 during the impregnation step by the beetle-killed material, given that increased SO_2 concentrations are well known to lead to reduced sugar recoveries (Schwald, et al. 1989). Again, sugar recoveries from the mixed substrate were between those for the beetle-killed and healthy.

The amount of glucose and mannose remaining in the insoluble fraction was similar for all three substrates, although the beetle-killed substrate was the only one that retained any galactose in the insoluble fraction (Figure 3-19). These results indicate that, in terms of solids recovery, the pretreatment conditions are well suited to the healthy and mixed substrates.

The amount of oligomeric sugars remaining in the water-soluble fraction for each sugar was not significantly different between the healthy, beetle-killed and mixed substrates (Figure 3-20). However, compared to the sugars in the original raw material, there was a slight loss of arabinose, xylose, and mannose from the healthy substrate compared to the beetle-killed (Figure 3-21). This loss of arabinose and xylose was also observed of after high-severity pretreatment of beetle-killed wood at in section 3.3.4.1, supporting the idea that the healthy wood was pretreated at an effectively higher severity than was the beetle-killed wood.

Table 3-7. Total sugar recovery from water-soluble and insoluble fractions after pretreatment of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine at medium (M) severity.

	Sugar recovery (% of original sugars)		
	BK-M	Healthy-M	Mix-M
Arabinose	78	53	66
Galactose	94	84	87
Glucose	100	100	100
Mannose	81	68	71
Xylose	73	67	68
Hexoses (weighted average)	96	93	94

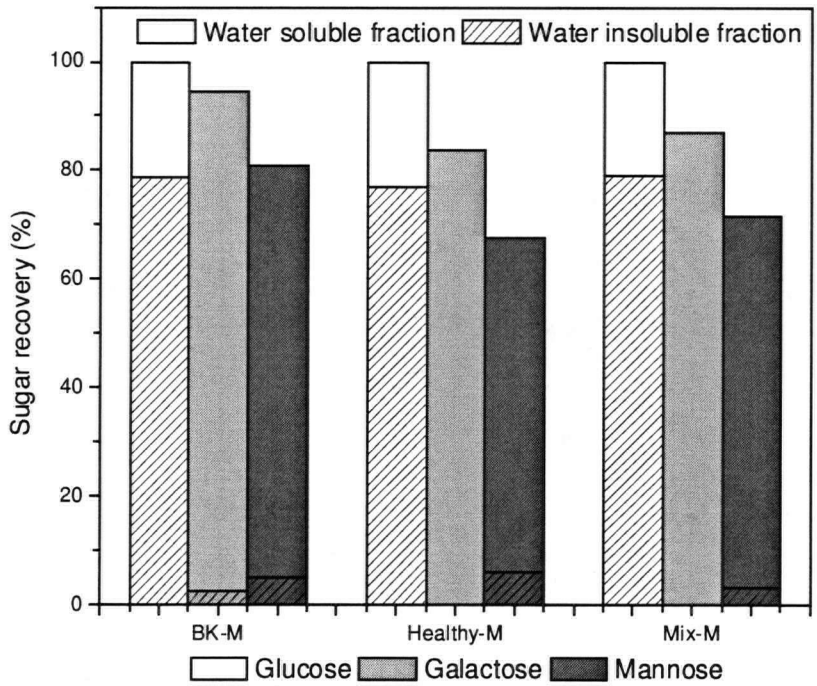

Figure 3-19. Glucose, galactose and mannose recovered in water-insoluble and soluble streams of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

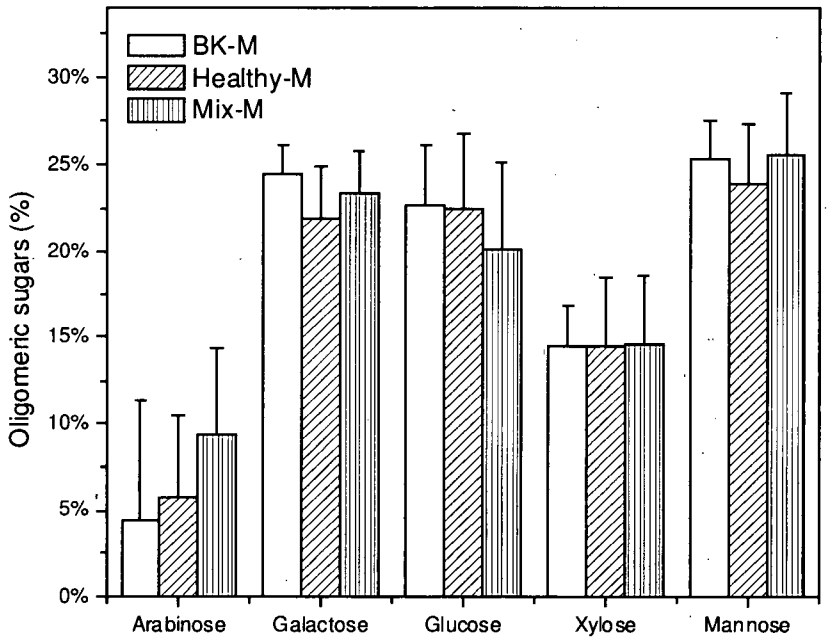

Figure 3-20. Amount of oligomeric sugars as a percentage of the total sugars in the water-soluble fraction of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

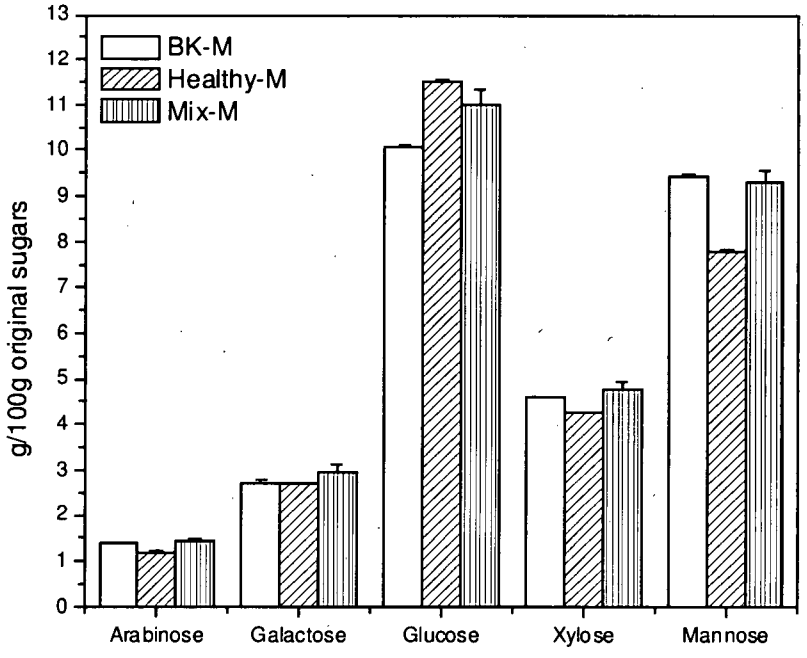

Figure 3-21. Sugar recovery from the water-soluble fraction of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

3.4.1.2 HMF and furfural analysis

The concentrations of HMF and furfural in the water-soluble fraction after pretreatment were not significantly different for the three substrates (Table 3-8). The same trend was observed after controlling for the amount of sugar in the starting material and dilution of the water-soluble fraction; the actual amount of inhibitors in each of the substrate was similar (Figure 3-22). This was somewhat unexpected, given the differences in sugar recovery observed, and that the beetle-killed wood pretreated at high severity showed similar sugar recovery as the medium-severity pretreated healthy wood but also showed higher HMF and furfural concentrations.

Table 3-8. Concentration of 5-hydroxymethyl furfural (HMF) and furfural in the water-soluble fraction (WSF) after pretreatment of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity. Deviation from the mean is shown in parentheses.

	Concentration in WSF after pretreatment (g/l)	
	HMF	Furfural
BK-M	2.23 (0.16)	1.04 (0.05)
Healthy-M	2.12 (0.11)	1.25 (0.05)
Mixed-M	2.38 (0.01)	0.97 (0.01)

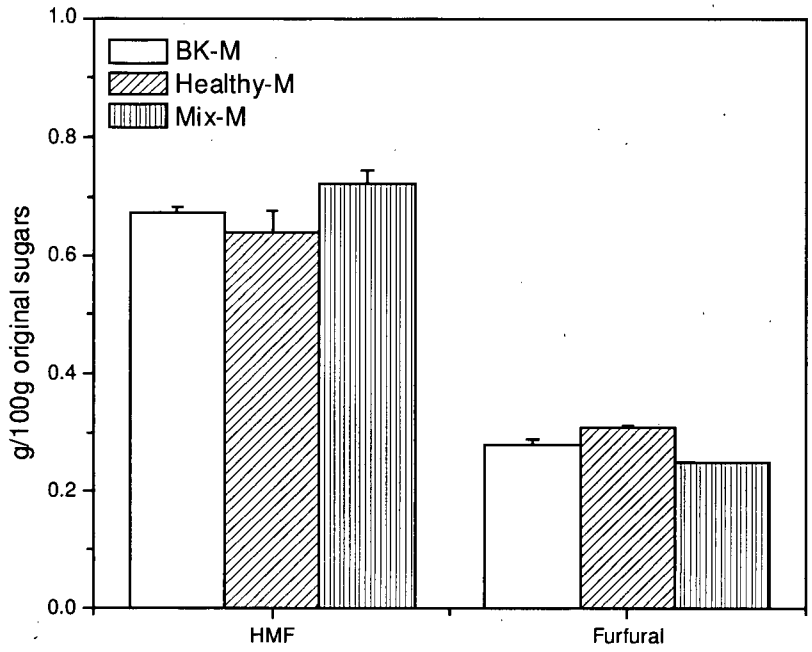

Figure 3-22. 5-hydroxymethyl furfural (HMF) and furfural concentration in the water-soluble fraction after pretreatment of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

3.4.2 Hydrolysis of healthy, beetle-killed and mixed substrates

Hydrolysis of the three substrates at 2% consistency revealed that, after 48 hours, there were no significant differences between the beetle-killed and mixed substrates, with the healthy substrate showing 14% lower conversion than the beetle-killed (Figure 3-23). This corresponds to 75, 66 and 77% of the original cellulose converted to glucose for beetle-killed, healthy and the mixed medium severity-treated pine. The increased hydrolytic conversion of the healthy and mixed substrates could be due to increased fines present in the beetle-killed wood. Fines are known to be higher in wood that has been extensively dried (Koch 1996; Thomas 1985), and have been shown to increase enzyme accessibility during hydrolysis due to increased surface area (Boussaid, et al. 2000; Mansfield, et al. 1999).

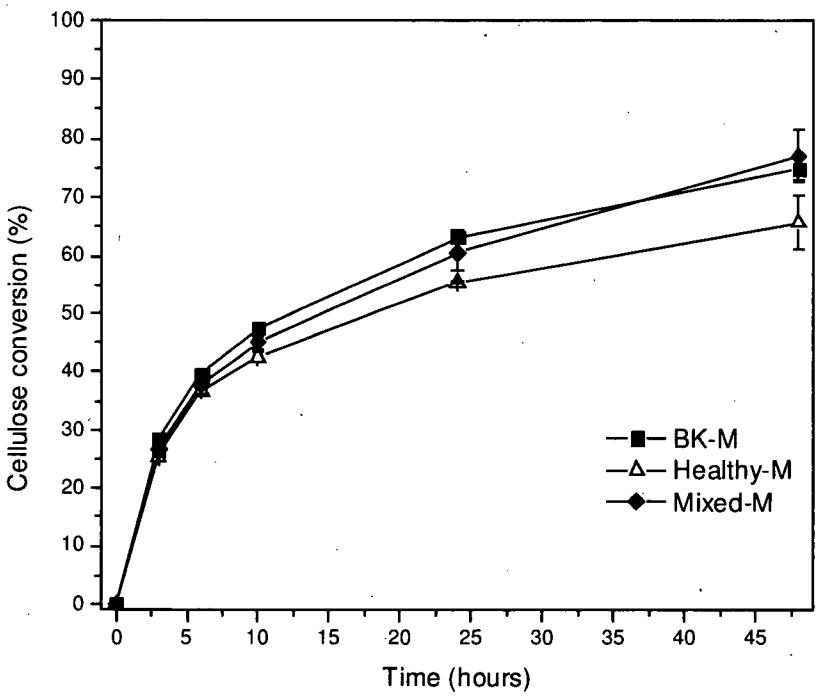

Figure 3-23. Conversion of cellulose to glucose during hydrolysis at 2% consistency of the washed solid fraction of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

3.4.3 Separate hydrolysis and fermentation of healthy, beetle-killed and mixed substrates

The hydrolysis portion of the SHF of beetle-killed, healthy, and mixed substrates provided 75, 62, and 74% conversion of cellulose to glucose after 72 hours of hydrolysis (Figure 3-24). These conversions (at 5% consistency) are only very slightly lower than those in the 2% consistency hydrolysis. Similarly, there was no significant difference in hydrolysability between the beetle-killed and mixed samples, with the healthy substrates showing 16% lower conversion than the beetle-killed. During fermentation, the rate of sugar consumption and ethanol production was slightly slower for the beetle-killed substrate, and this is reflected in the experimental ethanol yields of 75, 78 and 81% of theoretical for beetle-killed, healthy and mixed substrates respectively. However, because the beetle-killed raw material was lower in fermentable sugars and had better sugar recovery after pretreatment, similar overall yields of 72, 72 and 76% of the theoretical maximum ethanol yield from the raw material were obtained from the beetle-killed, healthy and mixed substrates.

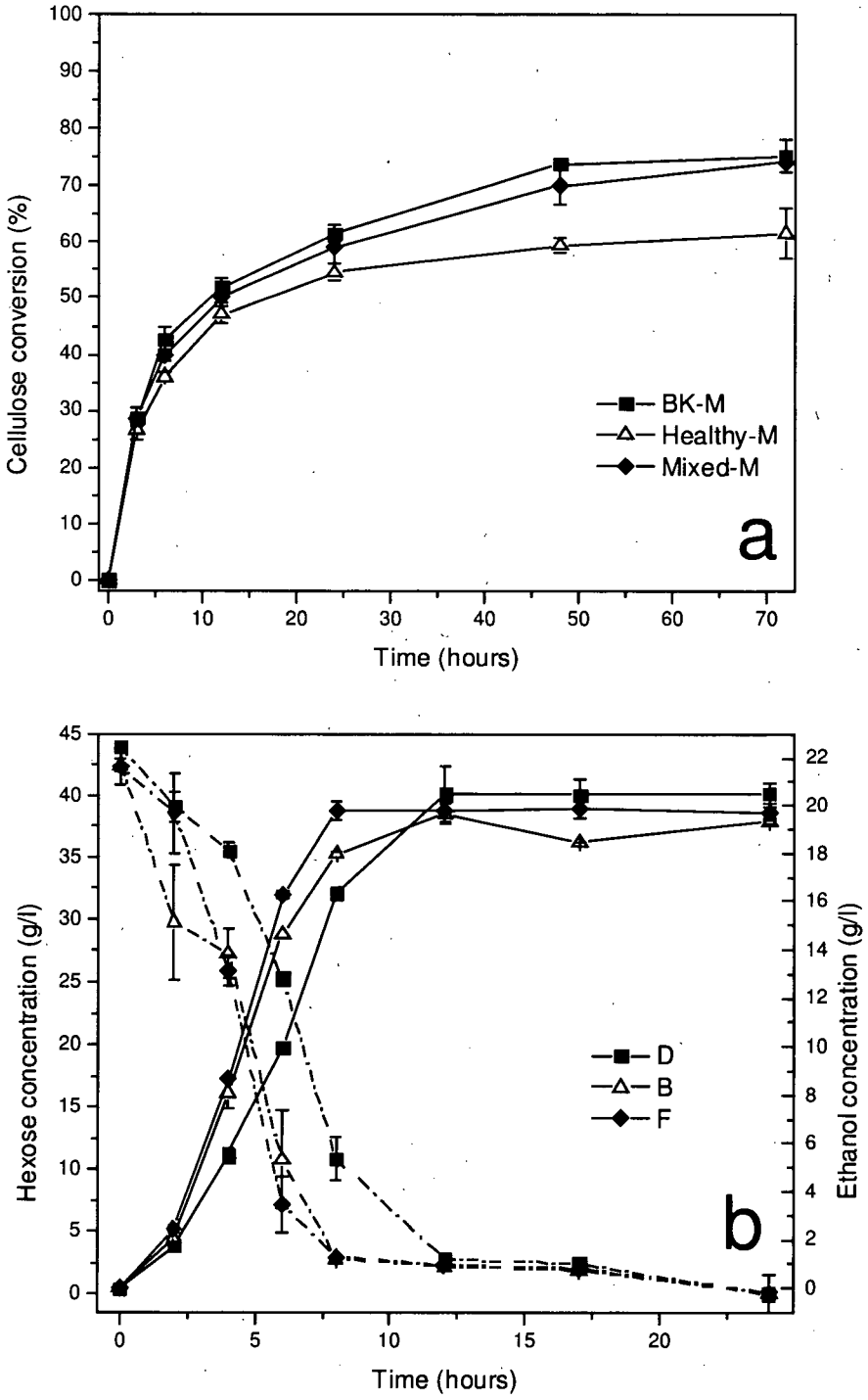

Figure 3-24. Hydrolysis (a) and fermentation showing hexose consumption (dotted lines) and ethanol production (solid lines) (b) during SHF of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

3.4.4 Simultaneous saccharification and fermentation of beetle-killed, healthy and mixed substrates

To reduce the longer residence time required by SHF, the SSF process developed in the previous section was applied to the healthy and mixed substrates. The rate of sugar consumption and ethanol production was lowest for the healthy substrate (Figure 3-25). Experimental ethanol yields after SSF were 80, 68 and 76% of the theoretical for beetle-killed, healthy and mixed. Taking into account sugar recoveries after pretreatment, this results in overall ethanol yields of 77, 63, and 71% of the theoretical ethanol yield from raw material for the beetle-killed, healthy and mixed lodgepole pine substrates, respectively.

Concentrations of furfural (Figure 3-26) and HMF (Figure 3-27) over the course of the SSF decreased as they were consumed. As in the previous section, during the 6-hour prehydrolysis there was a decrease of 39, 37, and 40% of HMF and 46, 48, and 49% of furfural for beetle-killed, healthy, and mixed substrates, respectively. After the prehydrolysis, the remaining furfural was completely consumed after 4 hours for all three substrates, while HMF took up to 24 hours to disappear. The HMF in the beetle-killed substrate was consumed at a slightly slower rate than the other substrates, though this does not seem to have affected the rate of ethanol production or hexose consumption.

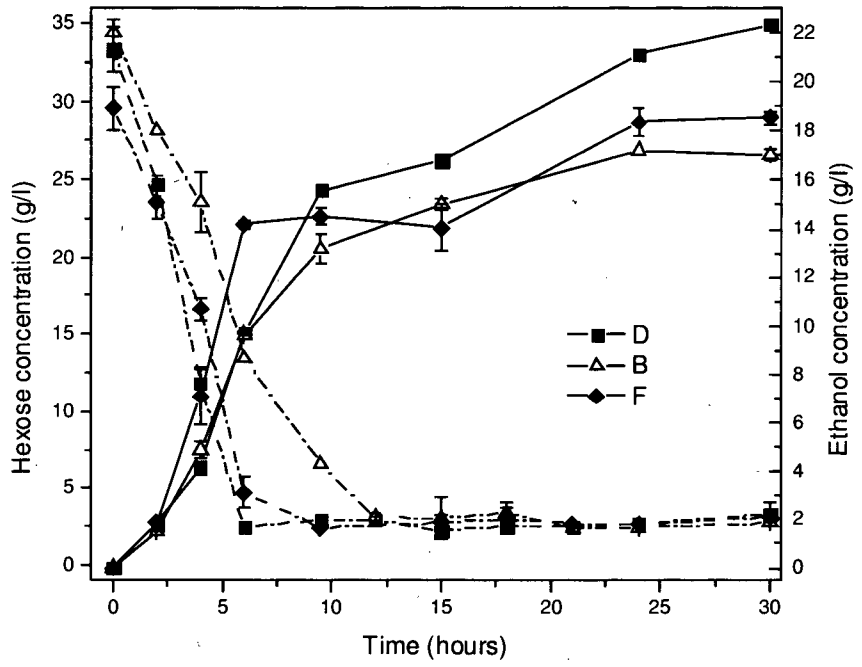

Figure 3-25. Hexose consumption (dotted lines) and ethanol production (solid lines) during SSF of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

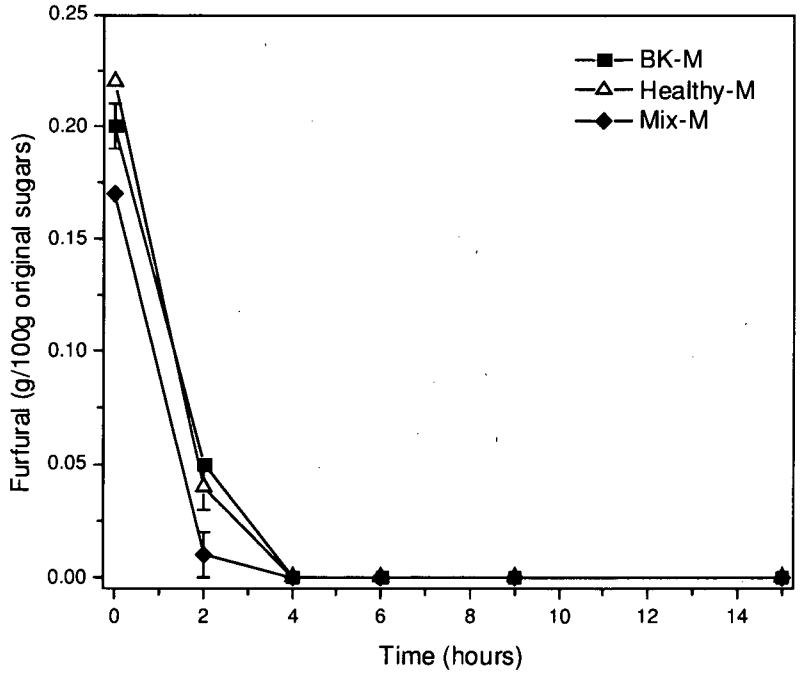

Figure 3-26. Consumption of furfural during simultaneous saccharification and fermentation (SSF) of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

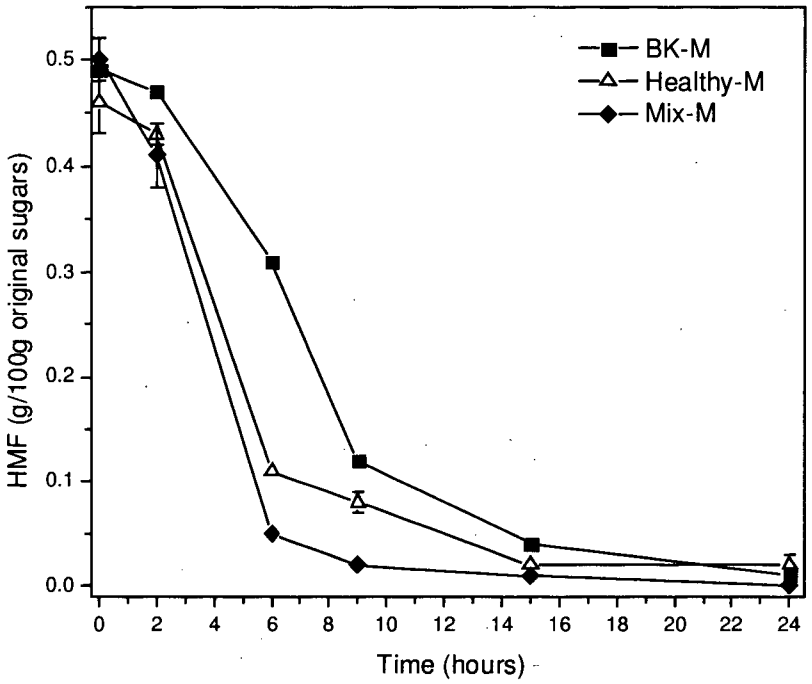

Figure 3-27. Consumption of 5-hydroxymethyl furfural (HMF) during simultaneous saccharification and fermentation (SSF) of beetle-killed (BK), healthy, and mixed (50/50 BK/healthy) lodgepole pine pretreated at medium (M) severity.

3.4.5 Conclusion

Although the original composition of the healthy wood did not vary considerably from the beetle-killed wood, after pretreatment the insoluble fraction of the beetle-killed substrate contained more cellulose and less glucose than did the healthy substrate. This is characteristic of milder-severity pretreatment, which was effectively the case given that the beetle-killed wood absorbed 20% less SO_2 during impregnation. The sugar recovery also reflected this, with higher recoveries of every sugar after pretreatment of the beetle-killed material, though HMF and furfural concentrations were similar for both. While a milder-severity pretreatment typically provides a substrate with reduced hydrolytic conversion, the beetle-killed substrate had 14% higher conversion than did the healthy. This was thought to be due to the increased amounts of the fines in the beetle-killed material, which improved the hydrolytic performance. The mixed substrate, as expected, generally had composition, recovery, and hydrolytic conversion values between those of the healthy and beetle-killed substrates.

Comparison of overall ethanol yields after SHF and SSF show that the highest yield, 77% of theoretical, is obtained after SSF of the beetle-killed material, over 20% higher than the yield achieved from the healthy substrate. The yield from the mixed substrate was in-between, as expected. There was no difference between the overall yields for beetle-killed and healthy substrates after SHF, with both generating 72% of the theoretical maximum ethanol yield. Curiously, the mixed material had a 5% higher yield after SHF, which may not be significant.

3.5 Comparison of healthy and beetle-killed pine pretreated at low, medium and high severity conditions

Beetle-killed lodgepole pine pretreated at medium severity provided higher ethanol yields after SSF than did healthy wood pretreated under the same conditions. In order to see whether this would be the case at other pretreatment conditions, healthy lodgepole pine was also pretreated at low and high severity and the resulting substrates subjected to SSF in order to further compare beetle-killed and healthy wood.

3.5.1 Sugar recovery, HMF and furfural analysis

Sugar recovery after pretreatment showed a predictable decrease as severity increased for all sugars (Table 3-9). Less than 40% of the starting arabinose and xylose were recovered. Compared to the pretreated beetle-killed material, sugar recoveries were an average of 20% lower for healthy wood at every severity level, indicating that the pretreatment conditions for healthy wood are more severe than for beetle-killed wood, likely as a result of the reduced SO_2 concentration.

Table 3-9. Total sugar recovery from water-soluble and insoluble fractions after pretreatment of healthy lodgepole pine at low (L), medium (M), and high (H) severities.

	Sugar recovery (g/100g original sugars)		
	Healthy-L	Healthy-M	Healthy-H
Arabinose	72	53	39
Galactose	92	84	63
Glucose	100	100	92
Mannose	76	68	46
Xylose	84	67	36
Hexoses (weighted average)	100	93	81

After pretreatment, the HMF and furfural concentrations in the water-soluble fraction increased with severity (Table 3-10), with the HMF concentration in the high-severity pretreated sample almost four times higher than in the low-severity material. Furfural

also increased two-fold from the low to high severity-pretreated samples. Both furfural and HMF concentrations were significantly higher after pretreatment at high severity than the corresponding beetle-killed substrate.

Table 3-10. Concentration of 5-hydroxymethyl furfural (HMF) and furfural in the water-soluble fraction (WSF) after pretreatment of healthy lodgepole pine at low, medium, and high severities. Deviation from the mean is shown in parentheses.

	Concentration in WSF after pretreatment (g/l)	
Severity	HMF	Furfural
Low	0.98 (0.00)	0.65 (0.02)
Medium	2.12 (0.11)	1.25 (0.05)
High	3.84 (0.02)	1.31 (0.04)

In relation to the amount of sugars in the starting material, there was not a significant increase in the total amount of HMF from low to medium severity, but the amount was almost doubled after pretreatment at high severity (Figure 3-28). The amount of furfural increased evenly with increased severity (Figure 3-29). Compared to the beetle-killed material, there was slightly less HMF in the healthy wood pretreated at low severity, but this amount increased at high severity. At low severity there was less furfural in the healthy material compared to the beetle-killed substrates, but at medium and high severity the amount of furfural was higher for the healthy substrate.

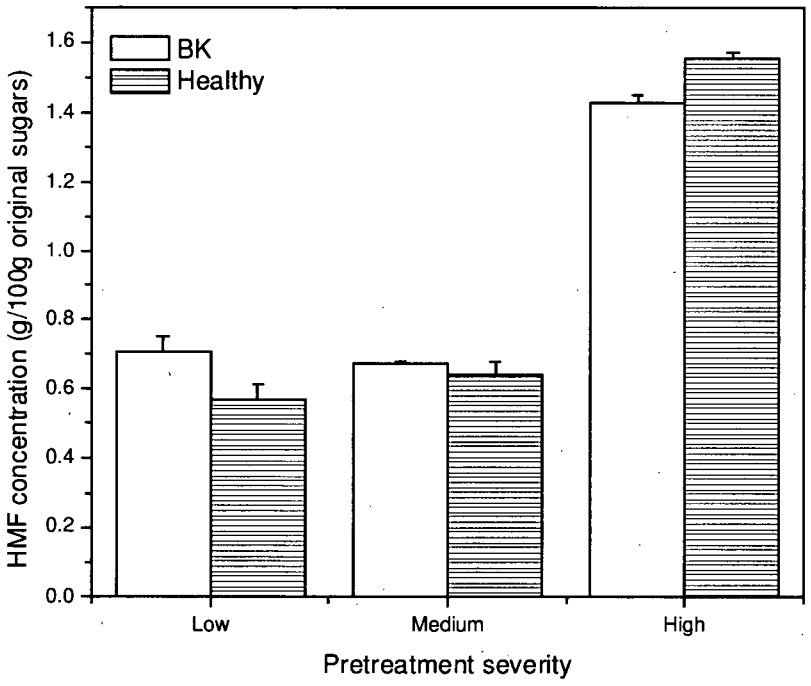

Figure 3-28. 5-hydroxymethyl furfural (HMF) concentration in the water-soluble fraction after pretreatment of healthy lodgepole pine at low, medium and high severities.

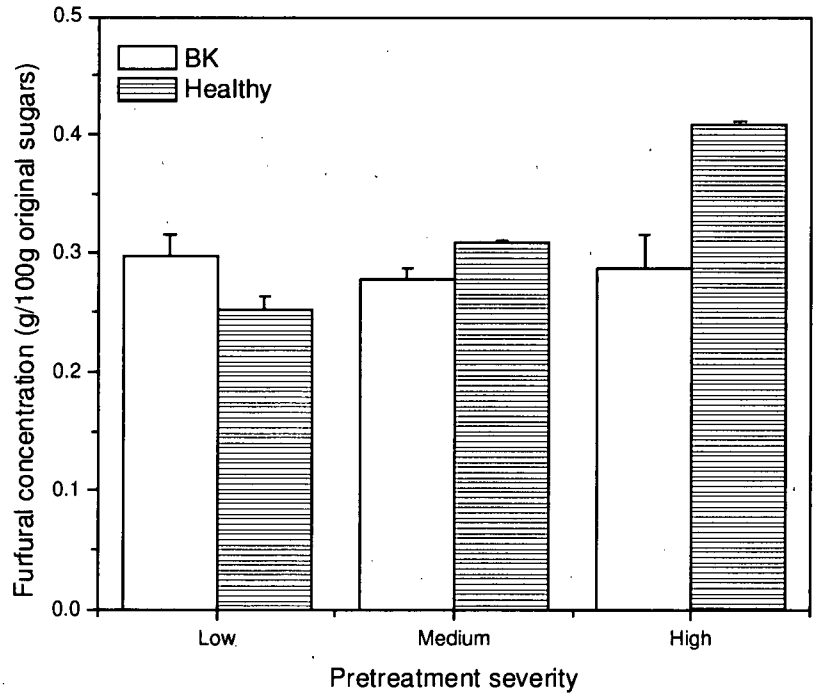

Figure 3-29. Furfural concentration in the water-soluble fraction after pretreatment of healthy lodgepole pine at low, medium and high severities.

3.5.2 Ethanol yield after simultaneous saccharification and fermentation

After SSF of the three substrates generated from low, medium and high severity pretreatment of healthy lodgepole pine, it was apparent that there were significant differences between the samples (Figure 3-30). The high severity-pretreated material had the slowest ethanol production rate, and 10 g/l of hexoses remained unconsumed after 30 hours. This poor fermentation was likely due to high concentrations of HMF and furfural present in the water-soluble fraction. The low-severity-pretreated material had the fastest ethanol production rate, but the final concentration was not as high as the others, likely due to incomplete hydrolysis. The medium severity-pretreatment resulted in the highest ethanol concentration, as well as the highest overall ethanol yield. Experimental ethanol yields for low, medium and high severity-pretreated substrates were 54, 68, and 61% of theoretical based on pretreated material, while overall yields were 52, 63, and 49% of theoretical based on sugars available in the raw material.

Compared to the beetle-killed substrates, the overall ethanol yields from healthy wood were lower for every pretreatment severity assessed (Figure 3-31). It is apparent that the medium severity pretreatment provides the highest ethanol yields after SSF for both beetle-killed and healthy wood, confirming the robustness of this condition. The 77 and 63% overall yields obtained from healthy and beetle-killed pine pretreated at medium severity are similar or higher than the 52% of theoretical ethanol yield reported by Bollok et al. (2000) for SSF of steam pretreated spruce or the 66% of theoretical reported by Stenberg et al. (2000a).

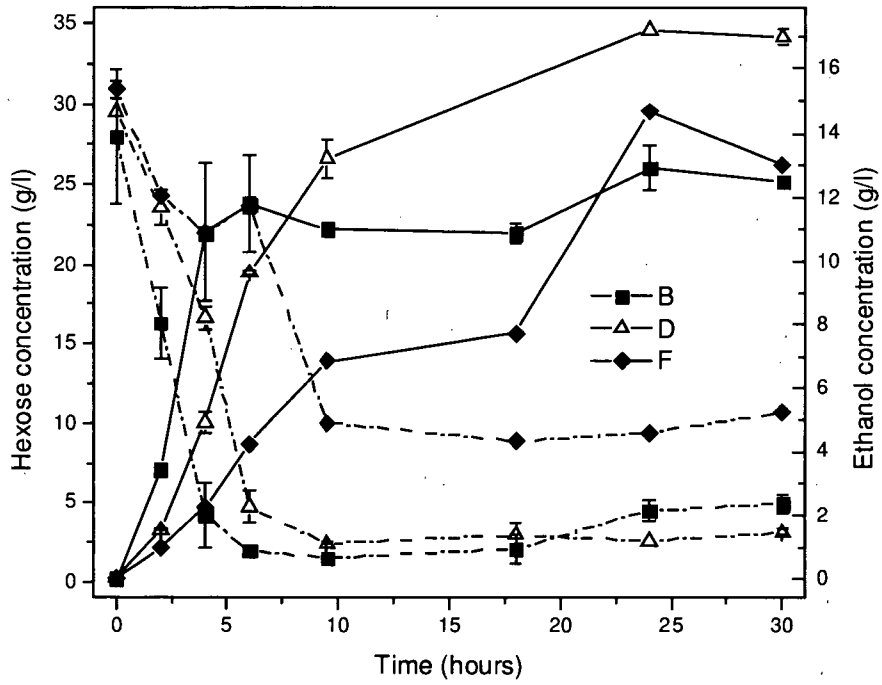

Figure 3-30. Hexose consumption (dotted lines) and ethanol production (solid lines) during SSF of healthy lodgepole pine pretreated at low (L), medium (M) and high (H) severities.

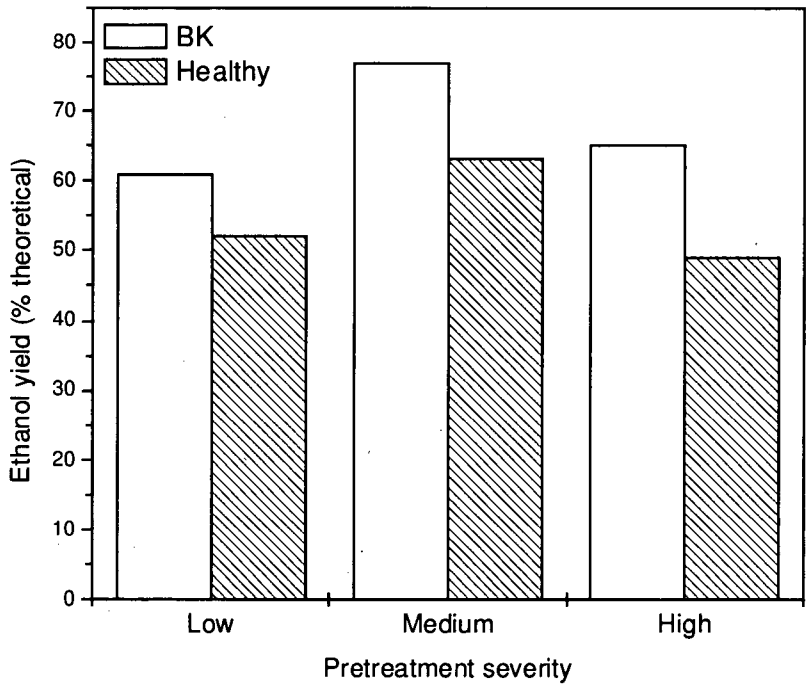

Figure 3-31. Comparison of overall ethanol yields after SSF of beetle-killed (BK) and healthy lodgepole pine pretreated at low, medium and high severities.

3.5.3 Conclusions and implications

Healthy lodgepole pine pretreated at low and high severity exhibited similar characteristics as the medium-severity treated substrate when compared to beetle-killed wood pretreated at the corresponding severities. Sugar recovery was, on average, 20% lower at all three severities compared to beetle-killed wood, although only the high-severity pretreated healthy substrate contained significantly higher HMF and furfural concentrations than the corresponding beetle-killed material. This increased concentration of inhibitors probably contributed to incomplete hexose consumption during SSF of the high-severity pretreated material. Overall ethanol yields after SSF for all three healthy substrates were 15-25% lower than the yields achieved when using the corresponding beetle-killed substrates. This was likely a combined result of increased sugar recovery, increased hydrolysability and lower levels of fermentation inhibitors.

3.5.3.1.1 Differences between bioconversion of beetle-killed and healthy substrates

The superior ethanol yields produced by the beetle-killed wood at every pretreatment condition could be a result of two factors occurring during pretreatment: reduced uptake of SO_2 during the impregnation, and increased physical degradation of the wood fibres. A key difference between the feedstocks is that the beetle-killed samples were cut from standing dead trees, while healthy samples were still alive when harvested. It is well known that drying of trees after death leads to pit aspiration and consequent resistance to permeation by gases (Comstock and Côté 1968; Petty and Puritch 1970). This would explain the reduced uptake of SO_2 during pretreatment, and since acid is the primary cause of sugar degradation during steam pretreatment, this reduced uptake leads to 20%

higher sugar recoveries for beetle-killed wood compared to healthy. Another result of increased drying is degradation of wood fibres and an increase in fines (Koch 1996; Thomas 1985). Increased fines in the pretreated material are know to have higher surface area and increased pore volume, allowing higher enzyme adsorption and increasing the rate and efficiency of hydrolysis (Boussaid, et al. 2000; Mansfield, et al. 1999). This accounts for the 14% higher conversion of the beetle-killed substrate observed after 48 hours of hydrolysis. The combination of increased permeability and fibre degradation allows the pretreated material to have the unique advantage of both high sugar recovery and high hydrolytic conversion, both of which are important for obtaining high ethanol yields.

Although every effort was made to keep the samples consistent, there were some physical differences between the healthy and beetle-killed wood samples. The beetle-killed wood was cut from upper (3.5m from the ground) and lower (0.3m from the ground) sections of the tree, while the healthy wood was only cut from the lower section. It has been shown that sugar concentration increases slightly with tree height in healthy wood but decreases with height in beetle-infested wood (Woo, et al. 2005). In addition, the proportion of sapwood to heartwood was not measured in either of the samples. Infested sapwood is reported to be more permeable than healthy, while healthy heartwood is more permeable than infested (Woo, et al. 2005). Another factor is that healthy trees were harvested from lower latitudes than the beetle-killed. Trees at higher latitudes have thinner sapwood and subsequently more heartwood, narrower growth rings, and denser heartwood (Hanna, et al. 1996). However, the difference in latitude is only 4° from the most northerly beetle-

killed tree to the most southerly healthy tree, which would probably preclude any significant differences.

It is well known that sapstaining fungi contribute to the desiccation and subsequent death of the tree, but in addition, these fungi and others might also modify the chemistry and structure of the wood. Differences in the chemical composition of beetle-killed and healthy wood are not great, with the biggest difference being 7% less glucose and 9% more lignin in the beetle-killed wood. Lieu et al. (1979) also found that there was no significant difference in the chemical composition of healthy and standing dead lodgepole pine. This is reasonable, since while sapstaining fungi do consume mannose and glucose, their primary energy source is lipids (Fleet, et al. 2001). While there has been a great deal of research on the effect of the beetle and fungus on lodgepole pine, the fact remains that there is a huge variation between trees in different areas and at different stages of attack. A variety of different fungi can colonize the trees after attack (Kim, et al. 2005), and moisture and other environmental factors can differ widely. These factors make it difficult to determine what the properties of a particular sample of lodgepole pine will be, and emphasize the importance of having a robust process which is effective on a feedstock with variable characteristics.

3.5.3.1.2 Economics

It has now been established that it is technically feasible to produce ethanol from beetle-killed lodgepole pine. However, in order for the process to be economically feasible, the cost of every aspect of the process must be considered. Minimizing of residence times, raw material costs, and energy usage are critical to producing a competitively priced

product. Utilizing SSF minimizes residence time as well as energy usage, while raw material costs for beetle-killed wood are lower than for healthy wood.

Wingren et al. (2005) showed that with steam-pretreated spruce, ethanol could be produced for $0.62 USD/litre. Since the cost of beetle-killed lodgepole pine is less than most other softwood feedstocks, including spruce, this price could be reduced further. Using the stumpage rates of $0.25 CAD/ m^3 assessed for low grades of beetle-killed lodgepole pine (BC Ministry of Forests November 2004) and delivery and processing costs of $25.71 CAD (Kumar, et al. 2005) a total raw material cost of $23 USD/$m^3$ can be calculated, equivalent to roughly $34 USD/oven dry tonne (ODT) (Ewanick 2006). Wingren et al. (2005) used a cost of $57 USD/ODT for their calculations. By reducing the raw material cost by 40%, the final product cost could be brought down to $0.57 USD/litre, an 8% reduction. In addition, the larger chip size used for lodgepole pine requires less milling than the spruce used in this economical analysis. In combination with reduced pretreatment time and temperature, energy costs can be reduced.

3.5.3.1.3 Process improvement

Ethanol yields after SSF of the medium-severity pretreated beetle-killed material were quite high, but it might be possible to achieve even higher yields by reducing the required residence time and/or yeast productivity. One means of accomplishing this is to grow *S. cerevisiae* on the water-soluble stream after pretreatment so that the yeast can adapt to the compounds present. Yeast cultivated on the pretreatment hydrolysate obtained from SO_2-catalysed steam explosion of spruce had an increased tolerance toward the inhibitors in the solution as well as higher ethanol productivity, in particular at high substrate loading during SSF experiments (Rudolf, et al. 2005). The yeast used in this thesis have

been adapted to spent sulphite liquor (SSL), which does not contain the same sugar or inhibitor concentrations as the water-soluble fraction generated during steam pretreatment. Another option would be to utilize a yeast variant that has been modified to consume galactose (Keating, et al. 2004a; Keating, et al. 2004b). While the Tembec T1 strain does consume galactose, the rate of uptake is slower, taking up to 48 hours to be completely removed. Increasing the rate of uptake could reduce the residence time while increasing the productivity.

4 CONCLUSIONS

Past work using Douglas-fir as a substrate at UBC showed that delignification was required to attain high hydrolytic conversion of pretreated substrates. However, this was not the case for other softwoods studied elsewhere, such as spruce and Radiata pine, from which high hydrolytic conversions could be achieved without the need for delignification. It was unclear whether these differences were a result of different pretreatment equipment and processes, or, more likely, due to differences in the chemical and physical characteristics of the wood. By investigating whether steam pretreated lodgepole pine could provide high overall ethanol yields, we examined the role that wood species plays in the softwood bioconversion process.

In order to ensure that the steam explosion process at UBC was on par with processes currently successfully pretreating softwoods, we collaborated with Lund University in Sweden. We compared spruce that was pretreated and shipped from Lund with the same raw substrate treated at UBC. We found that the composition of both substrates after pretreatment was similar to each other and to literature values for comparable substrates. Hydrolysis of the washed, water-insoluble fractions after pretreatment revealed that there was no significant difference in conversion after 48 hours. Based on these results, it was concluded that the UBC and Lund steam pretreatment processes produce similar substrates, and any past differences seen between pretreated spruce and Douglas-fir therefore appeared to be due to differences in the nature of the wood and not the pretreatment process.

Validation of the effectiveness of the UBC steam pretreatment process meant that we could optimize the conditions for beetle-killed lodgepole pine. Initially, seven conditions

were selected, ranging from the mild conditions used to pretreat Douglas-fir prior to delignification to the more severe conditions used for pretreatment of spruce. As pretreatment severity increased, there was a corresponding decrease in overall sugar recovery and fermentability and an increase in hydrolysability. After comparison of these factors, the seven conditions were narrowed down to three: low, medium and high severity. The low severity pretreatment (195°C, 7.5min, 4% SO_2) was chosen for its high recovery of fermentable sugars and fermentability, while the high severity pretreatment (215°C, 5min, 4.5% SO_2) generated a substrate with very high hydrolytic conversion and high sugar recovery. The medium severity (200°C, 5min, 4% SO_2) provided high sugar recovery, hydrolysability, and fermentability.

The low, medium and high severity-pretreated substrates were characterized for sugar, 5-hydroxymethyl furfural (HMF), and furfural content. As severity increased, the amount of hemicellulosic sugar recovered from the water-soluble stream decreased, while the amount of HMF increased as severity increased from low to medium to high. Separate hydrolysis and fermentation (SHF) of the three substrates provided very similar overall ethanol yields of 72-73% of theoretical based on fermentable sugars in the raw material. After development of the simultaneous saccharification and fermentation (SSF) method to include addition of nutrients and a prehydrolysis step, the overall yields were 61, 77 and 65% of theoretical for low, medium, and high severity-pretreated beetle-killed pine. Simultaneous saccharification and fermentation of the medium-severity pretreated material therefore provided a higher overall ethanol yield in one third of the time required for SHF, and was consequently chosen as the "optimum" condition.

The medium-severity conditions (200°C, 5 min, 4% SO_2) were subsequently applied to healthy and mixed (50/50 beetle-killed/healthy) lodgepole pine. In the next ten to twenty years, pine forests in British Columbia will likely consist of both healthy and beetle-killed trees. Considering this, it was essential to see whether the chosen pretreatment conditions were robust enough to also provide high ethanol yields from healthy and mixed wood. After pretreatment, sugar recovery was higher for all sugars in the substrate produced from beetle-killed material than from the healthy, although there were no major differences in HMF and furfural content. Hydrolysis of the water-insoluble fraction showed that the conversion after 48 hours was 14% higher for the beetle-killed substrate. After SHF, very similar overall ethanol yields were achieved: 72, 72, and 76% of theoretical from the original material from beetle-killed, healthy and mixed substrates. Prior to this, all results from the mixed substrate had, as expected, been in between those of the healthy and beetle-killed substrates. Simultaneous saccharification and fermentation of the three substrates provided overall ethanol yields of 77, 63, and 71% of the maximum theoretical from beetle-killed, healthy and mixed substrates respectively, showing that the beetle-killed substrate has the ideal balance of fermentability and hydrolysability. However, the yields achieved from the healthy and mixed substrates were still comparable to literature values, so it can be said that the medium-severity pretreatment is robust.

To further compare the behaviour of healthy and beetle-killed lodgepole pine, healthy wood was pretreated at low and high severity in order to evaluate the overall yields after SSF. Sugar recoveries after pretreatment declined with increasing severity, and there was a corresponding increase in HMF and furfural concentrations as severity increased. At

high severity, significantly more HMF and furfural were generated than in the corresponding beetle-killed substrate. After SSF, the overall ethanol yields at all three severities were lower than those obtained from beetle-killed wood pretreated at the corresponding severities. However, the highest ethanol yield for the healthy wood was achieved at medium severity conditions, confirming the robustness of that condition.

The superior sugar recovery and ethanol yields achieved using beetle-killed wood are thought to be a result of an effectively milder-severity pretreatment due to reduced uptake of SO_2 prior to pretreatment. In addition, the wood may have increased fines, which are known to improve enzymatic accessibility and efficiency during hydrolysis. The reduced permeability and poor fibre quality of the wood could be due to a number of factors, such as the drying out of the tree prior to harvest, or effects of sapstaining fungi and other colonizing microorganisms.

This work shows that beetle-killed lodgepole pine is an attractive feedstock for bioconversion to ethanol. In addition to the high ethanol yields achieved, production of a value-added product from an otherwise low value material, and the environmental benefits of removing flammable dry material from the forest make this an even more promising feedstock. Furthermore, healthy as well as mixed beetle-killed/healthy lodgepole pine can be pretreated with the same conditions and provide comparable ethanol yields, indicating that the process is sufficiently robust to be effective on a variable feedstock. Without requiring delignification, lodgepole pine has proven to be an attractive new prospect for bioconversion of wood to ethanol using SO_2-catalysed steam pretreatment.

5 FUTURE WORK

The use of higher consistency solids during SSF has been shown to increase the final yield of ethanol (Varga, et al. 2004). Using the 5% solids in this work as a starting point, SSF at 6-15% consistency could be investigated. Scaling up of the SSF process to the 1 litre fermentors available at UBC might increase the maximum consistency possible and/or result in higher ethanol yields because of pH, oxygen and temperature monitoring and maintenance as well as improved mixing efficiency. Ideally, the entire slurry after pretreatment (~16% solids) could undergo SSF, eliminating the need for separation of the water-soluble and insoluble streams and subsequent water washing of the solids. However, this would likely require longer residence times and high enzyme loading, so the economic feasibility would also need to be evaluated.

A comparison of beetle-killed and healthy lodgepole pine pretreated using both SO_2-catalyzed steam explosion and the organosolv process would be of interest. The cellulose fraction produced during the organosolv process is free of lignin and highly hydrolysable, and could be thought of as a "model substrate" (Pan, et al. 2005). By comparing the hydrolysability of the organosolv substrate to the steam-exploded substrate, the efficacy of the steam pretreatment could be evaluated. In addition, it could be determined whether the differences observed in hydrolytic performance between healthy and beetle-killed wood were due to the steam pretreatment process or the nature of the wood.

Further work could also examine the reasons why the beetle-killed wood provided higher ethanol yields. The effect of dryness could be investigated by comparing the bioconversion of dead, standing trees of another species to their healthy counterparts. The effect of the fungus could be explored by inoculating half of a sample stand of healthy

lodgepole pine trees with bluestain fungus, and comparing the bioconversion of the two feedstocks over time at different stages of fungal colonization. Similarly, to verify that the results presented in this thesis were due to fundamental differences in healthy and beetle-killed trees and not due to variability in the site type or gross tree characteristics, the experiments could be repeated using a larger sample size of trees with a full statistical analysis.

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